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# Characterization of Dihydroflavonol Reductase and Analysis of Condensed Tannin Accumulation in Response to Wounding and Herbivory in Trembling Aspen *(Populus tremuloïdes)*

by

Darren James Peters

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

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Dan Pet

2866 Robinson Street
Regina, Saskatchewan, Canada
S4S 1T8

Sept 26/2002

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Characterization of Dihydroflavonol Reductase and Analysis of Condensed Tannin Accumulation in Response to Wounding and Herbivory in Trembling Aspen (*Populus tremuloides*) submitted by Darren James Peters in partial fulfillment of the requirements for the degree of Master of Science in Plant Biology.

Dr. C. Peter Constabel

Dr. Gregory J. Taylor

Dr. Janusz J. Zwiazek

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**Abstract**

In order to study condensed tannin accumulation induced by wounding and herbivory, a dihydroflavonol reductase (DFR) cDNA was isolated from trembling aspen (*Populus tremuloides* Michx.). Bacterial overexpression confirmed that this cDNA encoded a functional DFR enzyme. Southern analysis indicated that *DFR* is likely a single copy gene in the aspen genome. *DFR* expression was induced after 24 h in both wounded and unwounded leaves on mechanically wounded aspen trees. DFR enzyme activity and condensed tannin concentrations also increased following wounding in wounded and unwounded aspen leaves. Wounding also induced the expression of other genes involved in the phenylpropanoid pathway. Feeding by forest tent caterpillar (*Malacosoma disstria* Hbn.) and satin moth (*Leucoma salicis* L.) larvae, and methyl jasmonate treatment all strongly induced *DFR* expression. This research suggests that the induction of condensed tannins, compounds known to be important in defense against herbivores, is mediated by the increased expression of *DFR* and other phenylpropanoid genes.

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**List of Abbreviations**

| | |
|---|---|
| Abs | absorbance |
| ATP | adenosine-5'-triphosphate |
| bp | base pairs |
| BSA | bovine serum albumin |
| CaMV | Cauliflower Mosaic Virus |
| cDNA | complementary deoxyribonucleic acid |
| CTAB | hexadecyltrimethylammonium bromide |
| df | degrees of freedom |
| DNA | deoxyribonucleic acid |
| dNTP | deoxynucleoside-5'-triphosphate |
| EDTA | ethylenediaminetetraacetic acid |
| EST | expressed sequence tag |
| FTC | forest tent caterpillar |
| FW | fresh weight |
| h | hours |

| | |
|---|---|
| IPTG | isopropylthio-β-D-galactoside |
| kD | kiloDaltons |
| LB | Luria-Bertani medium |
| LPI | leaf plastochron index |
| min | minutes |
| MOPS | 3-(*N*-morpholino)propanesulphonic acid |
| mRNA | messenger ribonucleic acid |
| n | number of samples |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| nos | nopaline synthase terminator |
| OD | optical density |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| p.f.u. | plaque forming units |
| RNA | ribonucleic acid |

| | |
|---|---|
| RNase | ribonuclease |
| rRNA | ribosomal RNA |
| SDS | sodium dodecyl sulphate |
| sp | species |
| U | units |
| UV | ultraviolet |
| v:v | volume per volume |
| w:v | weight per volume |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactoside |

# 1. Introduction

## *1.1 Overview of Plant Defense*

Most plants are damaged by herbivores at some stage in their life cycle. As a result, plants have evolved mechanisms to limit, impede or overcome herbivore damage by developing biochemical, physiological and morphological defenses. Many plants have constitutive defenses, which are always expressed in the plant. Additionally, plants exhibit induced defenses that are activated by herbivore damage (reviewed in Constabel, 1999 and Ananthakrishnan, 1999). Induced defenses differ in their speed of induction (rapid, short term or delayed, long term) and effectiveness against different herbivores. Carbon and nitrogen resources in the plant are diverted from growth and reproduction, and used to produce defensive compounds that provide induced resistance to many pests and pathogens. Wound-induced proteins and secondary metabolites are two major classes of defense compounds that plants produce to deter further herbivore damage. An overview of these defense compounds, as well as aspects of their regulation and signaling, is discussed in this section.

### *1.1.1 Protein-Based Defenses*

Protein-based defenses have been extensively studied, especially with respect to protease inhibitors. Genes encoding protease inhibitors have been shown to be induced in response to wounding in many plants (Ryan, 1990). These proteins are proposed to exhibit a defensive function by inhibiting proteolytic enzymes in the herbivore gut, which leads to oversecretion of digestive enzymes, such as trypsin, and a net loss of essential sulphur-containing amino acids. Ingestion of high concentrations of protease inhibitors

leads to inhibited growth in some insect pests (Broadway and Duffey, 1986). The majority of protease inhibitors studied in plants are inhibitors of serine proteases, though inhibitors of cysteine proteases, aspartic proteases, and metallo-proteases have been found (Ryan, 1990). The first studies of protease inhibitors were done in tomato leaves and found that gene expression and accumulation of serine protease inhibitors increased in wounded tissue within hours of damage, and that this activation occurred systemically in other undamaged parts of the plant (Green and Ryan, 1972). In trembling aspen (*Populus tremuloides* Michx.), three serine protease inhibitor genes were isolated and found to be induced by wounding, herbivore damage and methyl jasmonate treatments (Haruta *et al.*, 2001a). Furthermore, cysteine protease inhibitors, or cystatins, have been studied in legumes and cereals, and genes encoding cysteine protease inhibitors were found to be induced following wounding and methyl jasmonate treatment in soybean (Botella *et al.*, 1996).

In addition to protease inhibitors, oxidative proteins have been widely studied with respect to plant defense (reviewed in Constabel, 1999). Oxidative enzymes demonstrate defensive properties by having an anti-nutritive effect and degrading or modifying essential nutrients in the insect diet through oxidation. Phenol oxidases, peroxidases and lipoxygenases are all oxidative enzymes that are associated with stress responses in plants. Specifically, the protein polyphenol oxidase is proposed to be important in plant defense against insects (Felton *et al.*, 1989). Polyphenol oxidase produces highly reactive *o*-quinones from monophenolic and *o*-diphenolic compounds by catalyzing an oxidation reaction with molecular oxygen (Constabel *et al.*, 1996). *O*-quinones can reduce the nutritive value of protein by cross-linking and modifying the

sulphydryl and amine groups of proteins through alkylation, which prevents digestion and assimilation of amino acids by insect herbivores (Felton *et al.*, 1992). In hybrid poplar (*Populus trichocarpa* x *P. deltoides*) and trembling aspen, polyphenol oxidase gene expression is induced by wounding, herbivore damage and methyl jasmonate treatment in both damaged and systemic leaves (Constabel *et al.*, 2000; Haruta *et al.*, 2001b).

Another group of defensive proteins that have insecticidal activity are the lectins, which are carbohydrate-binding proteins that are proposed to interfere with digestive processes by binding to the surface of the epithelial cells of the insect midgut (Peumans and van Damme, 1995). The GS-II legume lectin from *Griffonia simplicifolia* was shown to significantly delay the development time of cowpea weevil (*Callosobruchus maculatus* F.) (Zhu *et al.*, 1996). Furthermore, the insecticidal activity of GS-II legume lectin was functionally linked to the ability of the lectin to bind carbohydrates, specifically *N*-acetylglucosamine (Zhu-Salzman *et al.*, 1998).

Protein-based defenses in plants have been shown to be effective in deterring herbivory by insect pests. Additionally, plants produce many secondary metabolites that also have a defensive function, and together with protein-based defenses, these compounds function as a multi-faceted defense response that protects the plant from a variety of pests and pathogens.

### *1.1.2 Secondary Metabolite Defenses*

Secondary metabolites are important components of the plant defense response. Plants produce a very large diversity of low molecular weight compounds known as secondary metabolites, which are not required for physiological processes in normal

growth and development, and whose biosynthesis is restricted to select plant groups. Secondary metabolites have a wide variety of functions including attractants for pollination and seed dispersal, and protection from abiotic and biotic stresses (Pichersky and Gang, 2000). Some secondary metabolites are produced as effective defenses against fungi, pathogens and microbes (phytoalexins) while other compounds function in defense against herbivores. In many cases, herbivores carry microbes to the damaged site, which stimulates the production of phytoalexins as a component of the plant response to herbivory. Secondary metabolites may be present as constitutive defenses, induced defenses or both in some plant species, and mainly act in defense as feeding deterrents, toxic compounds, or precursors for physical barriers. Secondary metabolites that have a role in defense against herbivores, pests and pathogens include the cyanogenic glycosides, glucosinolates, alkaloids, terpenoids, and phenolics (Bennett and Wallsgrove, 1994).

A widely distributed group of compounds with obvious defense-related functions is the cyanogenic glycosides, which are derived from aromatic and branched-chain amino acids (Halkier and Moller, 1991). The feeding deterrent activity of cyanogenic glycosides is apparently due to their hydrolysis by β-glucosidase after ingestion by the herbivore, resulting in the release of cyanide, which is a universal respiratory poison.

The glucosinolates are variable compounds that contain sulphur and nitrogen groups, and are produced mainly by plants of the order Capparales. Glucosinolates may have defensive properties due to their degradation by the enzyme myrosinase after disruption of plant tissue by herbivores, resulting in the production of isothiocyanate, nitrile and thiocyanate compounds, which may deter insect feeding. Most studies have

focused on glucosinolate production in oilseed rape (*Brassica napus* L.), which has been shown to be induced by insect attack and wounding (Koritsas *et al.*, 1989; Bodnaryk, 1992).

Alkaloids are toxic, nitrogen containing compounds that are usually derived from amino acids. These compounds are commonly found in Leguminosae, Liliaceae, and Solanaceae plants, and have been shown to be effective feeding deterrents against a number of herbivores. Resistance to the Colorado beetle (*Leptinotarsa decemlineata*) was found to be related to the concentration of the alkaloid demissine in the potato species *Solanum demissum* (Harborne, 1988).

One major group of structurally diverse secondary metabolites that is probably universal in the vascular plant kingdom is the phenolics. Phenolics are derived from aromatic amino acids produced by the shikimic acid pathway, and are characterized by possessing one or more aromatic rings bearing hydroxyl substituents. Furthermore, most phenolics are conjugated to sugar residues or other chemicals which stabilize the structure. Many phenolics that are induced by abiotic and biotic stresses are synthesized via the phenylpropanoid pathway. Phenylpropanoid compounds are induced by low temperature, high light, UV light, nutrient deficiencies in nitrogen, iron and phosphate, and pathogen and herbivore attack (Dixon and Paiva, 1995). Increases in the concentration of phenylpropanoid compounds likely occur due to the induction of enzymes involved in the synthesis of these compounds (Hahlbrock and Scheel, 1989). Additionally, many of these compounds are also present constitutively in tissues of some plant species, and induction of these compounds due to stress is usually specific to a plant species and plant tissue. An overview of phenylpropanoid metabolism is presented later.

*1.1.3 Regulation and Signaling in Plant Defense*

Though many plants exhibit constitutive defenses, induced defenses are important in deterring further infection and herbivore damage, and these defenses are regulated so that they are specifically expressed under conditions of biotic stress. The physical and chemical changes in a plant that are associated with pathogen infection and herbivore feeding are usually regulated by changes in gene expression. Genes encoding enzymes that are involved in the production of defensive proteins and phytochemicals are stimulated to varying degrees by damage, and increased gene expression usually results in the increased production of the defensive chemical. For example, phenolic compounds have been shown to accumulate in accordance with the transcriptional activation of genes encoding the enzyme phenylalanine ammonia-lyase (PAL), the first enzyme of the phenylpropanoid pathway (Hahlbrock and Scheel, 1989). Multiple forms of PAL are known to exist in many plant species, and their genes are induced differently by different stresses.

Intraplant signal transduction is involved in mediating systemic resistance in the plant by the increased production of defensive chemicals throughout the entire plant, even when damage is localized (reviewed in Walling, 2001). Plants produce a diverse array of signals, which are generated rapidly at the site of damage and travel through the plant. Jasmonic acid, a lipid-derived compound, is proposed to be an important signaling molecule that accumulates rapidly in injured plant tissue and induces the expression of many defense genes (Creelman *et al.*, 1992). Treatment with methyl jasmonate, a volatile methyl ester of jasmonic acid, has been shown to induce the accumulation of

proteinase inhibitors in tomato plants (Farmer and Ryan, 1990). Jasmonates are components of the octadecanoid signaling pathway, a lipid-based pathway that involves the wound-stimulated release of linolenic acid from membranes. Released linolenic acid is converted to jasmonic acid, which acts as an intracellular signal that activates the production of protease inhibitors, and other plant defenses. The release of linolenic acid from membranes in tomato is proposed to be mediated by an 18-amino-acid peptide, called systemin, which appears to be produced at the site of wounding and transported to distal parts of the plant (Constabel *et al.*, 1995). Systemin is translocated rapidly through the plant following wounding and has been shown to induce the synthesis of proteinase inhibitors (Pearce *et al.*, 1991). In addition to jasmonate signaling pathways, ethylene and salicylic acid production are also signaling methods that plants employ to enhance the production of defensive compounds. Thus, plants have a network of signals that are activated by herbivore damage and act together to result in intraplant systemic resistance.

In order to study plant defense responses, many experiments involve simulating herbivore damage by mechanically wounding the leaves of a plant. However, evidence suggests that mechanical wounding and herbivore damage have different effects on the plant defense response. In *Nicotiana sylvestris*, the application of herbivore and caterpillar regurgitants to mechanical wound sites was found to amplify the wound-induced accumulation of jasmonic acid (McCloud and Baldwin, 1997). Furthermore, the use of cDNA microarrays has demonstrated that gene transcript profiles are different for mechanically wounded leaves and leaves damaged by larvae of the cabbage butterfly (*Pieris rapae*) in *Arabidopsis* (Reymond *et al.*, 2000). Terpenoid volatiles involved in tritrophic interactions were only released from corn plants after caterpillar damage or the

application of caterpillar oral secretions to wound sites, and not after artificial damage (Turlings *et al.*, 1990). These volatiles act as long-range signals that attract parasites and predators of the herbivore, which acts as another line of defense in some plants. Thus, plants are capable of distinguishing between herbivore damage and mechanical wounding, and respond to these forms of damage differently.

In conclusion, plants exhibit intricate methods of conveying signals that alert the whole plant to wounding and herbivore damage. These signals are involved in regulating both protein-based and secondary metabolite defenses. An important secondary metabolite defense is the production of phenylpropanoid compounds, which are synthesized by enzymes that are induced by jasmonic acid-mediated signal transduction pathways (Gundlach *et al.*, 1992). Phenylpropanoid metabolism will be discussed in the next section.

### *1.2 Overview of Phenylpropanoid Metabolism*

The production of phenylpropanoid compounds has been widely studied with respect to plant defense. Phenylpropanoid compounds include the phenolic acids, lignins, coumarins, furanocoumarins, stilbenes, flavonoids, isoflavonoids, and condensed tannins (Dixon and Paiva, 1995).

The phenylpropanoid pathway has been extensively studied, and the chemistry and enzymology of this pathway has been established through biochemical and mutant analysis. All phenylpropanoid compounds are initially synthesized from the amino acid phenylalanine, with the first step in the phenylpropanoid pathway being the deamination of phenylalanine catalyzed by the enzyme phenylalanine ammonia-lyase (PAL). PAL is

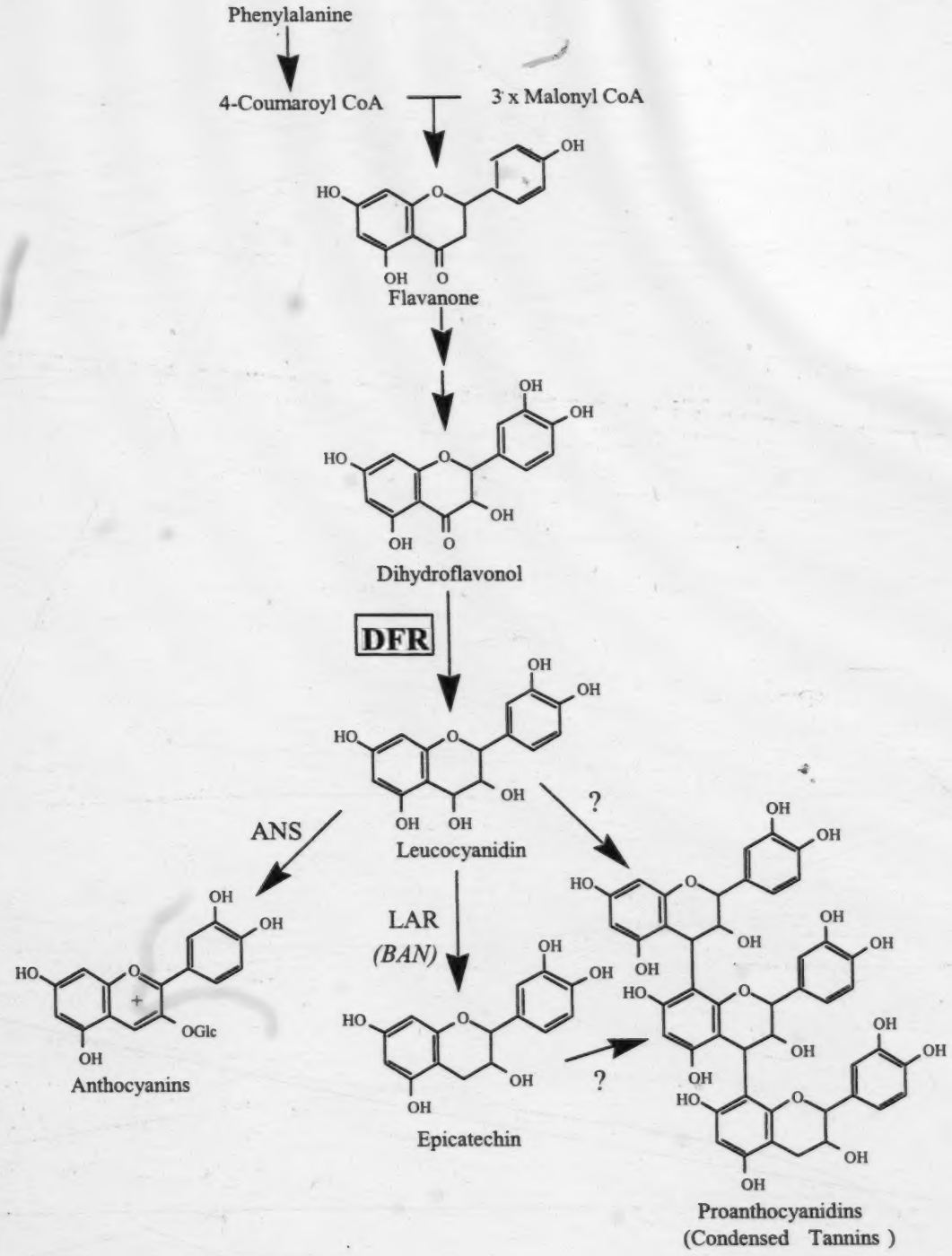
the major branch point enzyme between primary metabolism (phenylalanine from the shikimate pathway) and secondary metabolism (Hahlbrock and Scheel, 1989). Transcriptional activation of *PAL* has been observed in wounded and fungal infected hypocotyls of bean, and this induction also occurred systemically in distant, uninfected tissue (Lawton and Lamb, 1987). Furthermore, wounding rapidly induced the transcription of *PAL* mRNA in the leaves of hybrid poplar and the bark of trembling aspen, though this wound induction was variable among different genotypes (Koch *et al.*, 1998; Thamarus and Furnier, 1998). The reaction catalyzed by PAL produces cinnamate, a simple 9C phenolic compound. Cinnamate is further metabolized by the enzyme cinnamate 4-hydroxylase (C4H) to *p*-coumarate, which can be further hydroxylated, methylated and dehydrated to lignins, coumarins and furanocoumarins. The enzyme 4-coumarate:CoA ligase (4CL) catalyzes the addition of a coenzyme A to *p*-coumarate. Coenzyme A esters are the activated substrates that are used for the biosynthesis of the flavonoids, as well as lignin monomers. The expression of *4CL* is induced by wounding and fungal elicitor treatment in parsley and transgenic tobacco (Ellard-Ivey and Douglas, 1996).

A large and diverse number of stress-induced phenylpropanoids are derived from the C15 flavonoid skeleton, which is synthesized by the enzyme chalcone synthase (CHS). CHS catalyzes the condensation reaction of *p*-coumaroyl-CoA with three molecules of malonyl-CoA, producing the C15 compound 4,2',4',6'-tetrahydroxychalcone which is further metabolized to a diverse and widespread class of compounds known as the flavonoids in most plant species (Figure 1). Transcription of genes encoding *CHS* was found to be activated both locally and systemically by

wounding and the application of exogenous methyl jasmonate in white spruce (*Picea glauca*), soybean and petunia (Richard *et al.*, 2000; Creelman *et al.*, 1992; Tamari *et al.*, 1995). Alternatively, in a number of plant species, the condensation of *p*-coumaroyl-CoA with three malonyl-CoA molecules produces the stilbenes, via the catalytic action of stilbene synthase. The phenylpropanoid 4,2',4',6'-tetrahydroxychalcone is usually rearranged by the enzyme chalcone isomerase (CHI) to 5,7,4'-trihydroxyflavanone, which are present as flavones in some plant species. In some legumes, 5,7,4'-trihydroxyflavanone is isomerized by isoflavone synthase to produce the isoflavonoids. The enzyme flavanone 3-hydroxylase (F3H) hydroxylates 5,7,4'-trihydroxyflavanone to produce the dihydroflavonols, which are present as flavonols in many plant species. Dihydroflavonol may be reduced by the enzyme dihydroflavonol reductase (DFR) to produce flavan-3,4-diols, which are used in the synthesis of anthocyanins and condensed tannins. Condensed tannins are large polymers of flavan-3,4-diols and reduced flavan-3-ols (catechin), which are produced by the activity of the enzyme leucoanthocyanidin reductase (LAR).

Phenylpropanoid compounds have a wide variety of functions and their synthesis is regulated in the plant. Phenylpropanoid metabolism is commonly induced by abiotic and biotic stress and gene expression studies have demonstrated that the genes of phenylpropanoid synthesis are controlled mainly at the level of transcription (Weisshaar and Jenkins, 1998). The most common phenylpropanoid in the plant kingdom is the monolignols, which are the basic components of lignin synthesis, namely *p*-coumaryl, sinapyl and coniferyl alcohols. Lignin is formed by the polymerization of the

Figure 1: Flavonoid biosynthetic pathway leading to the synthesis of condensed tannins (proanthocyanidins). Enzyme names are abbreviated as follows: dihydroflavonol reductase (DFR), anthocyanidin synthase (ANS), leucoanthocyanidin reductase (LAR). "?" represents the unidentified condensing enzyme that is proposed to polymerize leucocyanidins and catechins to produce the condensed tannin polymer.


Phenylalanine
4-Coumaroyl CoA
3 x Malonyl CoA
Flavanone
Dihydroflavonol
DFR
Leucocyanidin
ANS
LAR
(BAN)
?
?
Anthocyanins
Epicatechin
Proanthocyanidins
(Condensed Tannins )

monolignols, and helps maintain the rigidity of the vascular system and contribute to leaf and stem toughness (Hagerman and Butler, 1991). The rapid deposition of lignin in order to create a barrier against fungal pathogen invasion has been recognized as an active physical defense in some plants (Hahlbrock and Scheel, 1989). Additionally, monolignols may dimerize to form lignans, which are reactive defense compounds that inhibit the growth of bacteria and fungi.

The compound *p*-coumaric acid may be hydroxylated and modified to form a lactone ring structure in plants of the Umbelliferae and Rutaceae, giving rise to the coumarins and furanocoumarins. Furanocoumarins, such as psoralen, are toxic, antibiotic substances that are activated by UV light, and found to react with pyrimidine bases of DNA and interfere with transcription and DNA repair mechanisms (Harborne, 1988). Other *p*-coumaric acid-derived compounds that have phytoalexin properties are the stilbenes and the isoflavonoids, which accumulate in response to fungal infection (Hart, 1981; He *et al.*, 1998).

The flavonoids are phenylpropanoid compounds that are induced by both abiotic and biotic stress. These compounds are important in providing protection against UV-B radiation, and are apparent as pigments that attract pollinators and aid in seed dispersal. Flavonols, such as kaempferol derivatives, have been shown to be induced by UV-B light irradiation in *Arabidopsis*, and are found to attenuate UV radiation that may otherwise damage DNA through breakage and dimerization (Lois and Buchanan, 1994). Additionally, flavonols are induced by wounding in the stigmas of petunia (Vogt *et al.*, 1994). Anthocyanins are the main pigments responsible for the orange, red and blue

colours of flower petals and fruit, and their synthesis is induced by high light, low temperature and low phosphate conditions (Dixon and Paiva, 1995).

With respect to defense against herbivores, condensed tannins are polymeric flavonoid compounds that were first implicated as having general chemical properties that may confer a defensive function. The importance of tannins in defense was first reported in oak, where feeding by winter moth larvae (*Opheropthera brumata* L.) was observed to stop in mid-June when concentrations of tannins in oak leaves significantly increased (Feeny, 1970). The defensive nature of tannins was proposed to be due to the ability of tannins to complex with proteins and digestive enzymes in the larvae gut.

### *1.3 Overview of Tannins*

Tannins are defined as high molecular weight, water soluble, polyphenolic natural products that have a capacity to strongly bind with carbohydrates and proteins (Haslam, 1993). The amount and type of tannins present varies widely between plant species. Tannin extracts were originally used in the conversion of animal hide to leather due to the tendency of tannins to form complexes with collagen fibres. Tannins are recognized as astringents in fruits, wines and other beverages due to their ability to precipitate proteins and mucopolysaccharides in the mucous secretions, and are the responsible agent for hazes in beer. The two classes of tannins are the hydrolyzable tannins and condensed tannins (proanthocyanidins). Some plant species produce hydrolyzable tannins, which are polymers of the shikimate pathway-derived phenolic, gallic acid, and sugar molecules (usually glucose) (Hagerman and Butler, 1991). Hydrolyzable tannins are not found in the foliage of trembling aspen (*Populus tremuloides* Michx.) (Bryant *et al.*, 1987), an

economically important tree species used in the study presented in this thesis. On the other hand, trembling aspen does produce condensed tannins, which are flavonoid oligomers and polymers that occur in many plant species. In the plant cell, tannins are stored in the central vacuole at high concentrations (Stafford, 1988). Tannins are found in leaves, stems and roots of woody angiosperms, and needles, stems, roots and strobili of gymnosperms. Also, some herbaceous angiosperms contain tannins in the seed coat (Porter, 1988). In general, the leaves of fast-growing, pioneer trees appear to have higher concentrations of condensed tannins than slow growing tree species (Shure and Wilson, 1993).

Condensed tannins have been widely studied with respect to biochemical structure, biosynthesis, and function in defense. Many assays have been designed that analyze the quantitative amounts and qualitative properties of condensed tannins (Hagerman and Butler, 1989). The principles of these assays that allow for the measurement of condensed tannins are dependant on the chemical structure and properties of condensed tannins.

### *1.3.1 Condensed Tannin Structure*

The structure of condensed tannins has been determined (Creasy and Swain, 1965), and possible modes of chemical synthesis have been proposed. Typically, condensed tannin polymers consist of 2 to 50 or more proanthocyanidin units. The fundamental structural unit of condensed tannins is the flavan-3-ol nucleus, which is the lower or initial unit of the oligomer (Haslam, 1993). Flavan-3,4-diols, which are the upper or extension units, are successively added in a linear chain to the terminal unit by

interflavonoid carbon-carbon bonds between the 4 and 8 carbon positions (Stafford, 1990). Furthermore, extension units may be added by linkages between the 4 and 6 carbon positions, which creates branches in the condensed tannin polymer. Thus, condensed tannin chains are extended only at one end. This model of condensed tannin synthesis is supported by labeling experiments where ten-times more radioactivity was found in upper, chain-extending units compared to lower, terminal groups (Stafford *et al.*, 1982). Most condensed tannins are aglycones, though some have been found that are conjugated to sugars or prenyl groups. The condensation reactions that are involved in forming the condensed tannin polymer can occur non-enzymatically, though the plant cell probably possesses condensing enzymes that regulate the process (Stafford, 1990). The average molecular weight and degree of polymerization of condensed tannins varies between plant species, within plant structures, and with development. In one study that characterized condensed tannins from 16 different plant species, the average molecular weight was found to vary from 1380 to greater than 4500 (Ayres *et al.*, 1997). In sainfoin (*Onobrychis viciifolia* Scop.) foliage, condensed tannin formation was found to be initiated early in leaf development, and the composition and content of condensed tannins changes during leaf expansion until the leaf is fully expanded (Lees *et al.*, 1995). Furthermore, condensed tannin polymers in sainfoin were found to increase in molecular weight and degree of polymerization as the leaf develops (Koupai-Abyzani *et al.*, 1993).

Each proanthocyanidin unit of condensed tannins is characterized as having A-, B-, and C-rings. The A-ring of the proanthocyanidin unit is typically hydroxylated at the 5 and 7 positions. The hydroxylation pattern of the B-ring distinguishes the proanthocyanidin unit as one of three different subgroups of proanthocyanidins. The

most common proanthocyanidin unit is the procyanidin (reduced-form of dihydroquercetin), which has a 3',4' hydroxylation pattern on the B-ring. Additionally, prodelphinidin (dihydromyricetin; 3',4',5' hydroxylation pattern on the B-ring) and propelargonidin (dihydrokaempferol; 4' hydroxylation pattern on the B-ring) units are found in condensed tannin polymers. The ratio of the relative amounts of each proanthocyanidin unit is variable among different plant species, as well as within different parts of the same plant and the stage of development of the plant. As leaves matured, the proportion of prodelphinidin in the condensed tannins of sainfoin foliage increased from 60% to 90%, illustrating that subunit composition is dynamic throughout development (Koupai-Abyazani *et al.*, 1993). In an extensive study, the ratio of procyanidin:prodelphinidin units was measured for 16 different woody plant species and found to range from 34:66 to 100:0 (Ayres *et al.*, 1997). The C-ring, which is the central heterocyclic ring, is typically hydroxylated at the 3 position.

Overall, the structure of condensed tannins has been elucidated and the presence of multiple phenolic groups suggests that these compounds are highly reactive and are able to associate with other metabolites. The reactive nature of tannins suggests that these compounds function in plant defense.

### *1.3.2 Function of Condensed Tannins in Defense*

The possible defensive role of condensed tannins has been extensively studied in many plant species. An overview of the mechanisms of tannin binding, as well as the role of condensed tannins in insect, mammalian, and ecological interactions will be presented. Also, evidence for condensed tannin induction in plants will be discussed.

*1.3.2.1 Mechanisms of Tannin Binding to Macromolecules*

The chemical structure and properties of condensed tannins allows for these compounds to have apparent defensive characteristics. The polyphenolic nature of tannins suggests that binding to macromolecules, such as carbohydrates and protein, occurs by hydrogen bond formation augmented by hydrophobic interactions (Haslam, 1993). The polarity of the multiple phenolic groups of tannins permits these compounds to form hydrogen bonds with other polar substrates, such as amide carbonyl groups in proteins. Furthermore, proteins rich in non-polar amino acids and the amino acid proline particularly tend to bind condensed tannins strongly, as these amino acids have a hydrophobic carbon-ring structure (Hagerman and Butler, 1991). Additionally, condensed tannins can participate in ionic bonds, which are formed when the phenolic groups are ionized in alkaline conditions to reactive phenolate ions that have a net negative charge. Though hydrogen bonding, hydrophobic interactions and ionic bonding may occur between tannins and proteins, the formation of oxidative covalent links between tannin and proteins appears to be the most important interaction in terms of the defensive activity of tannins.

Covalent bonding typically occurs due to the oxidation of tannins in the presence of enzymes, metal ions, or basic pH, which results in the formation of highly reactive *o*-quinones that rapidly react with other substances. The ability of *o*-quinones to donate an electron to molecular oxygen may result in the production of radical oxygen species, which are extremely dangerous to cells and may lead to enzyme inactivation, membrane lipid peroxidation, and DNA strand breakage (Appel, 1993). Condensed tannins are

expected to exert negative effects on insect herbivores that have an alkaline pH in their midgut by forming covalent bonds with dietary proteins and digestive enzymes in the insect midgut. The midgut of insect herbivores is the major site of nutrient digestion and absorption. Measurements of the physicochemical conditions in the midgut of many insect species has revealed variable conditions, such as redox potentials ranging from highly oxidizing to highly reducing, though most leaf-eating Lepidopterans have been found to have midguts with an alkaline pH (Appel, 1994). Alkaline conditions promote the autooxidation of phenolic compounds, which may then lead to the formation of covalent linkages with proteins. The hydrolyzable tannin, tannic acid, was added to artificial insect diets and found to undergo extensive oxidation in the midgut of tannin-sensitive larvae of the forest tent caterpillar (*Malacosoma disstria* Hbn.), which could result in covalent binding of tannins to dietary nutrients (Barbehenn and Martin, 1994). Covalent binding between tannins and digestive enzymes is expected to render the enzymes inactive and decrease digestion in the herbivore midgut. Low concentrations of condensed tannins from sorghum were shown to inhibit the digestive enzymes α-amylase and trypsin *in vitro* (Nguz *et al.*, 1998). Furthermore, tannins from three different forest tree species were shown to precipitate commercial β-glucosidase and esterase enzymes *in vitro*, though only esterase activity was inhibited due to precipitation (Juntheikki and Julkunen-Tiitto, 2000). In addition to protein binding, tannins appear to exert negative effects on insect herbivores by binding to the peritrophic membrane that lines the midgut. Oxidized condensed tannins are also able to form covalent linkages with surfactant phospholipids in the insect midgut, which may lead to increased gut surface tension, and lipid or dietary deficiencies in the insect (De Veau and Schultz, 1992). In general,

condensed tannins appear to have chemical properties that suggest these compounds may be effective in defense against herbivores.

*1.3.2.2 Defensive Function of Condensed Tannins against Insects*

The effects of ingested condensed tannins on insect herbivores have been studied in many plant species. To be effective deterrents, tannins must induce a negative response when consumed by herbivores, either through anti-nutritional effects or unpleasant, astringent sensations upon ingestion. Tannins may have anti-nutritional effects in herbivores by binding to dietary protein and decreasing its digestibility, resulting in a depressed herbivore growth rate and decreased food utilization efficiency. However, the role of condensed tannins in plant defense against herbivores appears to be variable, where tannins appear to exert a defensive function in some cases, though in others, they do not appear to have a defensive nature at all.

Condensed tannins have been studied in many plant-insect interactions, and have been found to negatively affect insect performance in some cases. In groundnut (*Arachis hypogaea*), condensed tannins appear to be an effective defense against herbivores as the concentration of condensed tannins was negatively correlated with groundnut aphid fecundity (*Aphis craccivora*) (Grayer *et al.*, 1992). Furthermore, the beech scale (*Cryptococcus fagisuga* Lind.) was found to preferentially infest beech (*Fagus sylvatica* L.) trees with low concentrations of condensed tannins in the inner bark, as condensed tannins reduce the nutritional quality of the bark (Krabel and Petercord, 2000). Finally, the soluble phenolic content of sorghum (*Sorghum bicolor* L.), which consists primarily of condensed tannins, is used as an indicator of resistance against the rice weevil

(*Sitophilus oryzae*) (Ramputh *et al.*, 1999). In most plant species, condensed tannins have variable effects against different herbivores. The southern armyworm (*Spodoptera eridania*) displayed suppressed growth rates when fed diets containing condensed tannins or hydrolyzable tannins from quebracho (*Schinopsis* sp.) trees, though tannins appeared to have no effect on the promethea silkworm (*Callosamia promethea*) (Manuwoto and Scriber, 1986). The variability in these studies could be explained by physicochemical conditions in the herbivore midgut, which may inhibit the oxidation and defensive activity of tannins. Additionally, the chemical structure of condensed tannins appears to be important in the activity against different insect herbivores. A landmark study was performed by Ayres *et al.* (1997), which revealed that condensed tannins from 16 different woody plant species differ widely in structure and activity against a variety of insect herbivores. In general, condensed tannins with a high molecular mass and a low ratio of procyanidin: prodelphinidin appear to be the most effective against herbivores. Since prodelphinidin units are the most highly hydroxylated proanthocyanidin units, condensed tannins containing a high quantity of prodelphinidin appear in general to have the highest antiherbivore activity. These tannins can undergo the most extensive oxidation in the insect gut. This study found that condensed tannins are effective defenses in some plant-herbivore interactions, though tannins do not appear to have a broad-spectrum defensive activity as would be predicted by their chemical structure and properties.

*1.3.2.3 Condensed Tannins and Mammalian Herbivores*

In addition to functioning as a chemical defense against insect herbivores, condensed tannins appear to have both defensive and beneficial characteristics when ingested by mammals. High concentrations of condensed tannins in forage plants are also detrimental to sheep and ruminants, and result in decreased voluntary intake, digestibility and adipose deposition (Aerts *et al.*, 1999). Furthermore, condensed tannins in blackbrush (*Coleogyne ramosissima*) inhibit food ingestion by snowshoe hares, though condensed tannins in bitterbrush (*Purshia tridentata*), which are structurally different, do not appear to affect the feeding of snowshoe hares (Clausen *et al.*, 1990). Additionally, some mammals appear to have adapted to condensed tannins by producing proline-rich proteins in the saliva, which bind tannins in the mouth and cause the complex to pass through the stomach without causing damage (Harborne and Williams, 2000). Thus, as has been observed for insect herbivores, condensed tannins also have a diversity of effects on mammalian herbivores, and the chemical structure of tannins is important in determining the defensive effectiveness against mammals.

In addition to their defensive characteristics, condensed tannins have been shown to have many beneficial effects when ingested by mammals. Condensed tannins are effective anti-microbial and anti-viral compounds, and may have anti-tumoral, antioxidant and anti-inflammatory properties when ingested by mammals (De Bruyne *et al.*, 1999). In sheep, condensed tannins are agronomically important as ingestion of forage containing 2-4 % condensed tannins is associated with increased availability and absorption of amino acids, which leads to increased body growth, wool growth and milk secretion (Aerts *et al.*, 1999). Furthermore, dietary tannins are important in preventing

pasture bloat by protecting protein from microbial digestion and reducing the stability of protein foam within the rumen. Condensed tannins have been shown to inhibit the proteolysis of casein and the large subunit of Rubisco by rumen microflora *in vitro*, thus the addition of dietary condensed tannins would result in increased protein absorption in ruminants, as protein is not lost through its conversion to ammonia by rumen microbes (Tanner *et al.*, 1994).

In conclusion, many lab-based studies have demonstrated that condensed tannins are correlated with both negative and positive effects on insect and mammalian herbivores. Furthermore, condensed tannins are expected to have important ecological roles in plant defense.

*1.3.2.4 Ecological Studies on Condensed Tannins in Defense*

The ecological role of condensed tannins in plant defense was first recognized by Feeny (1970) in studies of winter moth caterpillars feeding on the leaves of oak trees. Winter moth caterpillars feed on the leaves in spring, when the protein content in the leaves is high. In June, oak leaves were found to accumulate tannins to significantly higher concentrations, which changed the leaf palatability, and resulted in cessation of feeding by the winter moth caterpillars. This observation could not be accounted for by environmental factors or predators, and led to the first proposal that tannins deter insect feeding.

Furthermore, studies have assessed the role of tannins in plant-herbivore interactions in the tropics. The leaves of tropical trees are well defended by having low nutritional quality, high concentrations of secondary metabolites and high leaf toughness

(Coley and Barone, 1996). Many plants produce high concentrations of tannins, which are responsible for delaying the developmental time of insect larvae. However, tannins accumulate to high concentrations only after the leaves have completely expanded, and most of the herbivore damage occurs on young leaves before full expansion has taken place (Coley, 1983). Thus, many tropical plants have evolved mechanisms to neutralize herbivore damage to leaves by expanding their leaves more rapidly, or delaying greening until the leaves have reached maturity so that feeding insects are exposed to tannins and other metabolites.

In addition to having an ecological role in plant-herbivore interactions, condensed tannins are important in nutrient cycling and decomposition rates in soil ecosystems. Condensed tannins in leaf litter may complex with macromolecules in the soil and slow decomposition by delaying the breakdown and mineralization of detritus in the ecosystem (Baldwin *et al.*, 1983).

Condensed tannins are present constitutively in the foliage of many plant species, and both lab-based and field-based studies have suggested that condensed tannins may be important in plant defense. Studies focussing on whether condensed tannins may be present as an induced defense have also been performed, and evidence of induction would strengthen the case of condensed tannins being important in defense.

*1.3.2.5 Induction of Condensed Tannins by Wounding*

Wound-induced accumulation of condensed tannins has been observed in many plant species. Defoliation by the spearmarked moth leads to the production of higher concentrations of condensed tannins in Alaska paper birch (*Betula resinifera*), and these

increases may be important in the decline of spearmarked moth outbreaks (Bryant *et al.*, 1993). Defoliation by giraffes stimulates the production of condensed tannins in the leaves of damaged and nearby undamaged *Acacia nigrescens* trees (Furstenburg and van Hoven, 1994). Furthermore, condensed tannins are induced in the steles of cotton (*Gossypium hirsutum* L.) roots three to ten days after infection with *Verticillium dahliae* fungi, creating a toxic environment in the stele (Mace *et al.*, 1978). Lastly, mechanical wounding significantly increased the concentration of condensed tannins in leaves of karoo (*Ruschia spinosa*) shrubs (Stock *et al.*, 1993).

Thus, condensed tannins are induced in some plant species, and a genetic component is suspected to regulate this induction. Condensed tannin biosynthesis and the genetics of tannin regulation have been widely studied, and a better understanding of these aspects is important economically in the modification of condensed tannin concentrations for agriculture and pest resistance.

### *1.3.3 Mutant Analysis and Condensed Tannin Biosynthesis*

The enzymes of the general phenylpropanoid pathway and the flavonoid pathway synthesize condensed tannins, as described in section 1.2. In general, these enzymes are present constitutively in the plant. Genes that encode enzymes for each metabolic step of the phenylpropanoid pathway, leading to the synthesis of condensed tannins, have been identified by the isolation and analysis of mutants deficient in flavonoid synthesis. *Arabidopsis* has been used as a model plant in studying phenylpropanoid metabolism, and a total of 11 loci have been identified that are involved in flavonoid metabolism, as mutations in these loci lead to the reduction or absence of pigments in the seed coat

(Shirley *et al.*, 1995). Brown pigments in the *Arabidopsis* seed coat are mainly condensed tannins and flavonol derivatives. Seed coat mutations in *Arabidopsis* are collectively named *transparent testa* (*TT*) mutants, and a number of these mutants are specifically unable to synthesize condensed tannins in the seed coat. The absence of condensed tannins in the seed coat has been correlated with reduced seed dormancy, increased deterioration due to aging during storage, and permeability to tetrazolium salts, which indicates that seeds are more susceptible to exogenous stimulants resulting in early germination (Debeaujon *et al.*, 2000). *Arabidopsis TT* mutants have been well characterized, and the mutants analyzed include both structural and regulatory mutants of the condensed tannin pathway. Cloning of *TT* loci has revealed that single-copy genes encode almost all of the structural and regulatory enzymes of the general phenylpropanoid pathway in *Arabidopsis*.

In addition to *Arabidopsis*, mutant analysis of condensed tannin synthesis has been performed in barley (*Hordeum vulgare*), where the generation of condensed tannin-free barley would eliminate the formation of beer haze during brewing. More than 600 mutants have been isolated in barley, and these *ANT* mutants have also been well characterized. Structural and regulatory genes of the phenylpropanoid pathway have been assigned to the *ANT* mutations (pro*ant*hocyanidin-deficient) (Jende-Strid, 1991).

Of particular interest are *Arabidopsis* and barley mutants affected solely in condensed tannin synthesis. Most importantly, mutants have been identified that are deficient in the sequential NADPH-dependent reduction steps that occur in the latter part of the pathway, which are responsible for producing the extension flavan-3,4-diols and terminal flavan-3-ols that are polymerized into condensed tannins. NADPH-dependent

reducing activities are required for condensed tannin synthesis, and were first demonstrated in tissue cultures of the conifer, *Pseudotsuga menziesii* (Stafford and Lester, 1982). The enzymes dihydroflavonol reductase (DFR) and leucoanthocyanidin reductase (LAR) are responsible for these reducing activities, and mutants have been identified that are deficient in synthesizing functional DFR and LAR structural enzymes, and in genes that regulate the expression of *DFR* and *LAR*. DFR reduces dihydroflavonols and produces flavan-3,4-diols, which are the precursors of both anthocyanin and condensed tannin synthesis. The substrate specificity of DFR varies, as some enzymes from particular plant species do not reduce all three dihydroflavonols, or these enzymes may preferentially reduce one substrate over the others (see below). The latter reduction step of flavan-3,4-diols to flavan-3-ols (catechin) is unique to the condensed tannin pathway, and catalyzed by the enzyme LAR, which has been purified from sainfoin leaves (Tanner and Kristiansen, 1993).

The late stages of condensed tannin synthesis are still poorly understood. The precursors of condensed tannins, flavan-3,4-diols and flavan-3-ols, must be sequestered in the vacuole immediately after synthesis as these compounds are highly reactive and toxic in the cytoplasm. The *Arabidopis TT12* mutant is deficient in condensed tannin synthesis, and the mutation was cloned and found to encode a protein that appears to have 12 transmembrane segments, and similarity to prokaryotic and eukaryotic secondary transporters that belong to the MATE (multidrug and toxic compound extrusion) family (Debeaujon *et al.*, 2001). Thus, *TT12* may be required for the vacuolar sequestration of condensed tannin precursors and other flavonoids. After sequestration, condensed

tannins must be polymerized in the vacuole. Genes that encode condensing enzymes involved in the polymerization of condensed tannins have not been identified to date.

Transgenic studies that have investigated the regulation of condensed tannin synthesis have centred on the expression of dihydroflavonol reductase (*DFR*), which was used to modify concentrations of condensed tannins in plant tissues. Condensed tannin synthesis was down-regulated in "hairy root" cultures of *Lotus corniculatus* L. by transformation with a heterologous, partial *DFR* sequence under the control of the 35S CaMV promoter expressed in the antisense orientation (Carron *et al.*, 1994). Furthermore, concentrations of condensed tannins were found to both increase and decrease in transgenic *Lotus corniculatus* root cultures containing a heterologous, full-length *DFR* sequence under the control of the constitutive 35S CaMV promoter expressed in the sense orientation (Bavage *et al.*, 1997).

### *1.4 Dihydroflavonol Reductase*

Dihydroflavonol reductase is a key enzyme in the study of condensed tannins. The structure and regulation of DFR has been investigated in many plant species with respect to anthocyanin synthesis, and in a few plant species with respect to condensed tannin synthesis.

#### *1.4.1 Genomic Organization and Structure of DFR*

Genes that encode the enzyme dihydroflavonol reductase (DFR), which catalyzes the last common reaction in the flavonoid biosynthetic pathway leading to condensed tannins and anthocyanins, have been isolated and cloned from many plant species. *DFR*

has been found to occur as a single copy gene in *Arabidopsis*, grape, alfalfa, barley, snapdragon, tomato, and *Forsythia* (Shirley *et al.*, 1992; Sparvoli *et al.*, 1994; Charrier *et al.*, 1995; Kristiansen and Rohde, 1991; Martin *et al.*, 1985; Bongue-Bartelsman *et al.*, 1994; Rosati *et al.*, 1997) and multi-gene families have been characterized in *Zea mays*, *Petunia*, Japanese and common morning glories, strawberry and *Gerbera* (Bernhardt *et al.*, 1998; Beld *et al.*, 1989; Inagaki *et al.*, 1999; Moyano *et al.*, 1998; Helariutta *et al.*, 1993). Evidence that links condensed tannin biosynthesis to *DFR* expression has been provided by mutant barley and *Arabidopsis* plants that are unable to accumulate anthocyanins and condensed tannins due to inactivation of the *DFR* gene (Olsen *et al.*, 1993; Shirley *et al.*, 1995). The *ANT*18 locus in barley was found to contain the structural *DFR* gene, which encoded a functional DFR enzyme (Kristiansen and Rohde, 1991).

DFR enzymes have two major structural domains. One domain is the conserved NADPH-binding domain, which occurs near the N-terminus. The second structural domain in DFR enzymes determines the substrate specificity of the enzyme, which differs for different DFR enzymes from different plant species. A 26-amino acid region in the DFR enzyme has been identified as determining substrate specificity, as mutations in this region resulted in changes in the substrate specificity of *Petunia* DFR (Johnson *et al.*, 2001). In some plants, DFR is only able to reduce specific dihydroflavonols. This is probably due to the number of hydroxyl groups on the B ring of the dihydroflavonols, which bind to the DFR enzyme through hydrogen bonds at the substrate specificity site, though this is not the site of enzymatic action. Examples of DFR enzymes with narrow substrate specificity include English ivy (*Hedera helix* L.) DFR, which can reduce

dihydroquercetin but not dihydrokaempferol, resulting in the accumulation of only cyanidin and not pelargonidin anthocyanins (Murray and Hackett, 1991). Furthermore, DFR from *Cymbidium hybrida* is not able to efficiently reduce dihydrokaempferol, thus *Cymbidium* is unable to produce orange pelargonidin anthocyanins (Johnson *et al.*, 1999).

### *1.4.2 Regulation of Dihydroflavonol Reductase*

DFR is mainly regulated at the level of transcription, and the expression of *DFR* genes and DFR enzyme activity has been found to typically parallel the accumulation of anthocyanins in some studies (Beld *et al.*, 1989; Helariutta *et al.*, 1993; Moyano *et al.*, 1998). *DFR* appears to be predominantly regulated by factors that bind to *cis*-acting elements in the promoter region. A large family of transcription factors identified in plants that function in the control of phenylpropanoid metabolism are the MYB proteins, which have DNA-binding domains that can recognize specific target motifs in a DNA sequence (Martin and Paz-Ares, 1997). The light-inducible *MYB-P1* gene from *Perilla frutescens*, which encodes a protein with high similarity to regulatory, DNA-binding MYB proteins, was able to bind to the promoter of *DFR* and interact with a MYC-related transcriptional regulatory protein that is involved in the control of anthocyanin synthesis (Gong *et al.*, 1999). Furthermore, the *ANT*13 gene in barley encodes a regulatory protein that is required for the expression of *DFR*, and the accumulation of anthocyanins and condensed tannins (Meldgaard, 1992).

Recently, characterization of *Arabidopsis TT* mutants has lead to the discovery of regulatory genes that encode proteins required for the expression of *DFR* and condensed tannin accumulation. The gene *TT8* encodes a basic helix-loop-helix (bHLH) DNA-

binding protein that may bind to putative bHLH recognition sequences in the *DFR* promoter (Nesi *et al.*, 2000). Two other genes, *TTG1* (transparent testa glabrous 1) and *TT2*, encode a WD-repeat-containing protein and a R2M3 MYB protein respectively (Nesi *et al.*, 2001). All three of these genes, *TT2, TT8* and *TTG1*, are required for the normal expression of *DFR* during *Arabidopsis* seed development, though additional tissue-specific factors are involved in regulating condensed tannin synthesis as overexpression of *TT2* did not induce condensed tannin accumulation. Furthermore, all of these regulatory genes were also found to be required for the expression of the *BANYULS* (*BAN*) genes, which encodes a DFR-like protein that may have leucoanthocyanidin reductase (LAR) activity (Devic *et al.*, 1999). The *BAN* gene was identified from an *Arabidopsis* mutant that does not accumulate flavan-3-ols (catechins) in the seed coat, and cloning revealed that the *BAN* gene contains a conserved NADPH-binding region and is expressed specifically in the seed coat. The overexpression of *TT8* and *TT2* in *Arabidopsis* transgenics was found to induce *BAN* gene expression (Nesi *et al.*, 2001).

In addition to condensed tannins, DFR is required for anthocyanin synthesis. Anthocyanins are water-soluble flavonoid pigments that accumulate in the vacuole and serve to attract insects and animals for pollination and seed dispersal, and provide protection against UV radiation (Strack and Wray, 1989). After DFR reduces dihydroflavonols to flavan-3,4-diols, the enzyme leucoanthocyanidin dioxygenase (anthocyanidin synthase; ANS) converts the flavan-3,4-diols to anthocyanidins (Pelletier *et al.*, 1997). Leaves of trembling aspen have been shown to produce cyanidin-type anthocyanins in the fall, which results in the observed red and orange pigments (Chang *et*

*al.*, 1989). Anthocyanins have been shown to accumulate in response to wounding, cold and phosphate stress with accompanying increases in *DFR* gene expression or DFR enzyme activity (Tamari et al., 1995; Hasegawa et al., 2001; Dedaldechamp and Uhel, 1999). Some plants contain multiple *DFR* genes that encode differing DFR enzymes that may function specifically in either anthocyanin or condensed tannin synthesis.

Overall, DFR is a key enzyme in the synthesis of condensed tannins, and a good enzyme to study when monitoring changes in condensed tannin concentrations. This thesis details a study of *DFR* expression and condensed tannin accumulation in trembling aspen (*Populus tremuloides* Michx.), a forest tree species that is damaged by many insect herbivores. Trembling aspen (*Populus tremuloides*) is an economically important tree species that is widely used in the pulp and construction industry.

### *1.5 Overview of Trembling Aspen (Populus tremuloides) Herbivore Defense*

#### *1.5.1 Chemical Defense in Trembling Aspen*

Trembling aspen has a widespread distribution across North America, and grows on a variety of soils. Aspen is an early-successional species that colonizes areas that have been disturbed by fire, logging or other factors. Trees reproduce by both sexual and asexual means, and asexual suckering rapidly produces large stands of genetically identical clones. In many parts of its range, trembling aspen is susceptible to massive defoliation by a variety of insect herbivores such as the forest tent caterpillar (*Malacosoma disstria* Hbn.), large aspen tortrix (*Choristoneura conflictana* Walker) and gypsy moth (*Lymantria dispar* L.) (Ives and Wong, 1988). Populations of forest tent

caterpillars have been found to rise and fall in approximately 10-year intervals, and population outbreaks cause extensive defoliation in aspen forests (Duncan and Hodson, 1958). However, aspen trees are not uniformly susceptible to defoliation by insect herbivores, and the production of defense compounds appears to be important in deterring insect herbivores (Hemming and Lindroth, 1995). Defense against insect herbivores has been studied extensively in trembling aspen, especially in regards to the effectiveness of secondary metabolite defenses. Trembling aspen possesses both protein-based and biochemical (secondary metabolite) defenses. Protein defenses in aspen include Kunitz trypsin inhibitor proteins and polyphenol oxidase, which accumulate after mechanical wounding, herbivore attack and methyl jasmonate treatment (Haruta *et al.*, 2001a; Haruta *et al.*, 2001b). Foliar secondary metabolites of aspen that have a role in defense include the condensed tannins and phenolic glycosides. The foliar concentrations of phenolic glycosides and condensed tannins can vary widely among different aspen clones; up to 3.8- and 8.4-fold respectively as found in one study (Hwang and Lindroth, 1997).

Phenolic glycosides are salicyl alcohol-derived phenylpropanoids, which can be present at concentrations of up to 7% dry weight in aspen foliage (Lindroth and Hwang, 1996). There are four phenolic glycosides present in trembling aspen: salicin, salicortin, tremuloidin and tremulacin. These compounds have negative effects on larval performance of the forest tent caterpillar and gypsy moth, most likely by causing the formation of degenerative lesions in the insect midgut (Hemming and Lindroth, 1995; Hwang and Lindroth, 1997). Forest tent caterpillar larvae reared on diets containing 3.5% phenolic glycosides from aspen and low protein (1.0%) content displayed the

highest first instar mortality and development times in bioassays (Lindroth and Bloomer, 1991). Furthermore, the addition of esterase inhibitors potentiated the toxicity of phenolic glycosides, as esterases in the insect midgut are implicated in the detoxification of phenolic glycosides (Lindroth and Bloomer, 1991). Concentrations of phenolic glycosides appear to be affected by light and $CO_2$ concentrations as aspen trees grown in high light (855 $\mu mol\ m^{-2}\ s^{-1}$) and elevated $CO_2$ (696 $\mu L\ L^{-1}$) conditions displayed increased levels of phenolic glycosides, which reduced the performance of white-marked tussock moth caterpillars (*Orgyia leucostigma*) (Agrell *et al.*, 2000). Furthermore, the phenolic glycosides, salicortin and tremulacin, are inducible and have been shown to accumulate within 24 h in response to simulated herbivory in aspen (Clausen *et al.*, 1989).

Condensed tannins (proanthocyanidins) are found at concentrations of up to 18% dry weight in aspen leaves (Lindroth and Hwang, 1996). The amount and distribution of condensed tannins present in aspen foliage varies with age, season and nutrient availability. For the most part, condensed tannin concentrations have been observed to increase rapidly during leaf expansion, and remain constant or increase slightly for the remainder of the season (Lindroth and Hwang, 1996; Shure and Wilson, 1993). Growth conditions, such as nutrient and light availability, are important in determining the accumulation of secondary metabolites in aspen tissues. High concentrations of condensed tannins have generally been observed in conditions of nutrient stress, high light intensity and $CO_2$ enrichment. Condensed tannins were found at the highest concentrations in leaves from aspen trees grown on low-fertility soil (1.25 mmol $L^{-1}$ $NO_3^-$) compared to high-fertility soil (7.50 mmol $L^{-1}$ $NO_3^-$) (Kinney *et al.*, 1997).

Furthermore, condensed tannin concentrations also appear to be affected by light, as aspen trees grown in high light conditions (1209 ± 49 μmol $m^{-2}$ $sec^{-1}$) had higher concentrations of condensed tannins compared to trees grown in low light conditions (235 ± 15 μmol $m^{-2}$ $s^{-1}$) (Hemming and Lindroth, 1999). Interestingly, increased levels of condensed tannins in aspen foliage grown under high light conditions could be reduced by the addition of nutrients (7 g $L^{-1}$ 18:6:12 NPK fertilizer), indicating that light and nutrient availability interact to have a total affect on the accumulation of condensed tannins in aspen (Hemming and Lindroth, 1999). Furthermore, there is a large degree of genetic variation in aspen with respect to the amount of phenolic compounds produced, as there is a large between-clone and within-clone variation in condensed tannins, and some clones do not respond to changes in nutrient availability as others (Hemming and Lindroth, 1999).

*1.5.2 Aspen Condensed Tannins as an Herbivore Defense*

Variation in the effectiveness of condensed tannins as a defense against major insect herbivores of aspen is apparent in the literature. Data providing evidence that support or refute a putative role for condensed tannins in aspen defense have been reported. Differences in the literature could be a result of differences in experimental conditions or real variation to the extent that condensed tannins play a role in defense. High concentrations of condensed tannins have been shown to limit damage by a number of insect herbivores in fast-growing clones of aspen (*Populus tremula* x *P. tremuloides*) (Gruppe *et al.*, 1999). Furthermore, gypsy moth larvae and forest tent caterpillars were found to have poor performance on aspen grown in elevated $CO_2$ and high light-low

nutrient conditions respectively, as these trees displayed low concentrations of foliar nitrogen and high concentrations of condensed tannins (Traw *et al.*, 1996; Hemming and Lindroth, 1999). Insect herbivores showed a lower ability to efficiently convert digested food to body mass and displayed compensatory feeding when consuming foliage from aspen trees grown in elevated $CO_2$, which had higher concentrations of condensed tannins (Kinney *et al.*, 1997). Condensed tannins are expected to function by binding macromolecules and diluting nutrients in the larval gut, which results in compensatory feeding by the insect due to the inability to gain nutrients from the aspen foliage. In turn, compensatory feeding causes the insect to be exposed to more toxic chemicals in the aspen foliage, such as the phenolic glycosides, which have also been shown to negatively affect insect performance. Though there is support for condensed tannins in plant defense, other experiments have found no significant negative correlation between leaf tannin levels and herbivore performance (Hemming and Lindroth, 1995).

A role for condensed tannins in defense would be further supported by showing that condensed tannins are induced by wounding in aspen foliage. Recently, artificial defoliation was shown to increase the concentrations of condensed tannins in trembling aspen leaves, especially under conditions of low nutrient availability (Osier and Lindroth, 2001). Additionally, the synthesis of condensed tannins was also induced in young leaves of hybrid poplar (*Populus deltoides* x *P. nigra*) by jasmonic acid application, though gypsy moth herbivory did not induce tannins by 24 h after wounding (Arnold and Schultz, 2002). Clearly demonstrating that wounding induces both condensed tannin accumulation and the expression of the enzymes involved in synthesizing condensed

tannins would be consistent with condensed tannins having a defensive role in trembling aspen.

Thus, there is evidence that suggests condensed tannins in the leaves of trembling aspen are important defense compounds against insect herbivores. Preliminary data from work done by Constabel and Spence (unpublished data) indicated a strong inverse relationship between condensed tannin concentrations in aspen foliage and growth rates of forest tent caterpillar larvae. These findings led to the undertaking of this study.

### *1.6 Objectives of This Study*

Condensed tannins have been extensively studied in terms of their structure, biological effects on herbivores and biosynthesis, and the molecular biology of the phenylpropanoid pathway has been well defined. The work described in this thesis takes a molecular approach to the study of induced condensed tannin synthesis in trembling aspen (*Populus tremuloides*). The genus *Populus* is a good system for molecular studies in tree species, as it has a small genome size, is easily propagated, and has well-developed *Agrobacterium* transformation and regeneration protocols. Aspen in particular is ideally suited to the study of the molecular biology of condensed tannins, as aspen foliage contains high concentrations of condensed tannins, and aspen has been shown to have an inducible defense response. When this research began, condensed tannin induction by wounding or herbivory had not been reported for trembling aspen, though a recent paper has shown the wound-induced accumulation of condensed tannins in aspen (Osier and Lindroth, 2001).

Dihydroflavonol reductase (DFR) was chosen as a molecular tool to study the molecular aspects of condensed tannin synthesis in *Populus tremuloides*. DFR is the last structural enzyme of the condensed tannin pathway that has been cloned and confirmed to function in tannin synthesis. To date, no studies in any plant species have investigated the wound-induction of condensed tannins at the level of gene expression, and induced condensed tannin accumulation has not been previously connected with DFR induction. For this research, I planned to link increases in DFR expression and activity to induced condensed tannin synthesis. The objectives of this study were i) to clone and characterize the expression of DFR, a key enzyme in condensed tannin synthesis, and to demonstrate its induction by herbivory, ii) to confirm wound-induced condensed tannin accumulation in aspen leaves, and iii) to correlate increases in DFR gene expression with condensed tannin accumulation as well as the expression of other defense and phenylpropanoid genes. These findings would suggest that condensed tannins are an inducible defense in trembling aspen, and would provide insight into the regulation and signaling involved in induced condensed tannin synthesis.

## 2. Materials and Methods

### *2.1 Trembling Aspen Micropropagation and Growth Conditions*

Locally obtained trembling aspen (*Populus tremuloides* Michx.) genotypes were propagated *in vitro*. Four different genotypes (A2, G2, C1 and C4) were propagated by aseptic micropropagation techniques. Genotypes C1 and C4 originated from George Lake, Alberta, and genotypes A2 and G2 originated from Edmonton, Alberta. C1 and C4 were of particular interest as C1 had high constitutive concentrations of condensed tannins in the field, while C4 had low constitutive concentrations of condensed tannins in the field (Spence and Constabel, unpublished data). Tissue culture solutions and media were prepared as described (see Appendix 1) (Rajora, 1992). Initial aspen multishoot cultures were produced from dormant buds, which were surface sterilized and aseptically transferred to solid bud break medium (ACM-1) containing 20 mg $L^{-1}$ adenine sulphate and 0.5 mg $L^{-1}$ 6-benzylaminopurine (BAP) in Magenta GA-7 vessels (Dr. C. P. Constabel and J. Patton performed bud preparation and initial shoot preparation). Shoots were induced and propagated by transferring buds to shoot proliferation medium (ACM-2) containing 20 mg $L^{-1}$ adenine sulphate, 0.5 mg $L^{-1}$ BAP and 0.02 mg $L^{-1}$ naphthalene acetic acid (NAA). Aseptic multishoot cultures were maintained by subculturing shoots to fresh ACM-2 medium (Rajora, 1992). Rooting was induced by transferring shoots to medium (ACM-3) containing 0.5 mg $L^{-1}$ indole-3-butyric acid (IBA) and 0.02 mg $L^{-1}$ naphthalene acetic acid. Aseptic multishoot cultures were maintained in a MLR-350 Versatile Environmental Test Chamber (Sanyo Canada, Toronto, Ontario) at 25 °C under a 16:8 h light/dark regime. Rooting cultures were maintained in a University of Alberta

environmental growth chamber (Environmental Growth Chambers, Chagrin Falls, Ohio) at 22 °C under a 16:8 h light/dark regime.

In preparation for transfer to soil, four-month-old rooting cultures were acclimated by placing plastic wrap over the uncovered Magenta GA-7 vessel (Sigma-Aldrich, Oakville, Ontario) and allowing the plants to ventilate in room conditions over one week. Plants were transferred to autoclaved soil (Terra-Lite Redi-Earth, W.R. Grace, Ajax, Ontario) in 5-cm pots, and acclimated in the University of Alberta Biotron's environmental chambers (250 µmol $m^{-2}$ $sec^{-1}$) at 20 °C under a 16:8 h light/dark regime. Successfully acclimated plants were transferred to soil containing 10 mL 14-14-14 N-P-K controlled release plant fertilizer with micronutrients (Plant Products, Brampton, Ontario) in 15-cm pots. Routine experiments were carried out with 10-12 week-old plants, which had 25-35 leaves. Genotypes A2 and G2 were used in most experiments since these clones grew the best using the propagation techniques and growth chamber conditions described.

Aspen flower tissues were obtained locally during May 2000 from native trembling aspen trees growing in the Edmonton river valley, and individual flowers were dissected out of aspen catkins.

*2.2 Experimental Stress Treatments*

For experiments, leaf numbers were assigned based on the leaf plastochron index (LPI) method, which is a method that quantifies sapling growth rates based on the finding that leaves are initiated at relatively uniform rates when grown in a constant environment (Larson and Isebrands, 1971). All aspen trees used in experiments possessed at least 25

leaves; thus all trees had a leaf plastochron index of at least 25. Leaves were numbered from the apical meristem downward, beginning with the first developing leaf of lamina length 20-mm, which was designated as the index leaf (LPI 0). Mechanical wounding was performed to simulate herbivory by crushing the leaf margins three times at two-hour intervals using a hemostat. Time course experiments involved wounding six leaves on six aspen trees, and three wounded and three unwounded upper (systemic) leaves were harvested at the appropriate times. Leaves of LPI 16-19 on aspen plants were generally used as the wounded leaves while leaves of LPI 12-15 were the systemic leaves, since leaves within these ranges appeared to display the least variation with respect to condensed tannin concentration (see Results). Controls were taken before wounding by removing approximately one-quarter of an aspen leaf from each aspen tree with a razor blade.

Forest tent caterpillar experiments were conducted in 75 cm-high insect cages within the environmental chambers, and involved placing 12 fourth and fifth instar forest tent caterpillar (*Malacosoma disstria* Hbn.) larvae (obtained from Dr. A. Keddie, University of Alberta) on each aspen tree. The larvae were allowed to feed for three 20-minute intervals every 2 h. Satin moth (*Leucoma salicis* L.) larvae were collected locally from infested poplar trees, and leaf damage was induced by placing 20 fourth and fifth instar larvae on plants within insect cages in the growth chambers and allowing continuous feeding for 96 h. Undamaged aspen trees were also placed in insect cages as controls and leaves were harvested at the same time that leaves from damaged trees were harvested.

Plants were treated with methyl jasmonate (Bedoukian, Danbury, Connecticut) by exposing them to 20 µl of a 10% solution of methyl jasmonate in ethanol on a cotton wick in sealed glass aquariums (Haruta *et al.*, 2001a). Control plants were sealed in glass aquariums and exposed to 20 µl of a 90% ethanol solution on a cotton wick. Treatments were performed in the Biotron growth chambers.

For all experiments, harvested plant tissues were frozen in liquid nitrogen, ground using a mortar and pestle, and stored at –80 °C until analyzed.

### *2.2.1 Statistical Analysis*

Statistical analysis was performed on primary data from time course experiments that measured DFR activity and condensed tannin concentrations in control, wounded and systemic leaves.

For time course experiments measuring DFR activity, single factor ANOVA analysis was performed using Excel 2000 (Microsoft Corporation) software. In these experiments, three aspen plants were wounded, and control samples were taken by harvesting approximately one-quarter of each wounded aspen leaf from each tree. Control samples taken from the same tree were pooled for protein extraction. At each appropriate time point after wounding, one wounded and one systemic leaf was harvested from each aspen plant for individual analysis. For each time point, three independent measurements of DFR activity were used in ANOVA analysis for comparison with control measurements. ANOVA analysis was used to determine if the mean DFR activity from wounded and systemic tissue at each time point (n=3) was different from the mean DFR activity from control leaves (n=3).

For time course experiments measuring condensed tannin concentrations, single factor ANOVA analysis was performed using Excel 2000 (Microsoft Corporation) software. In these experiments, eight aspen trees were wounded, and control samples were taken from both systemic and wounded leaves (described in 3.6). Control samples of the same leaf age (LPI) were pooled for analysis. Sampling at each time point involved haphazardly selecting five of the eight wounded plants and harvesting one wounded leaf from each selected tree. This process was also independently repeated for systemic samples. Each wounded and systemic leaf sample was individually analyzed for condensed tannins. For each time point, five independent measurements of condensed tannins were used in ANOVA analysis for comparison with control measurements. ANOVA analysis was used to determine if the mean condensed tannin concentration in wounded and systemic tissue at each time point (n=5) was different from the mean condensed tannin concentration in leaves of the same age (n=4).

### *2.3 Cloning and Sequencing of DFR cDNA*

#### *2.3.1 Initial Cloning of DFR and Primary Screening of aspen cDNA library*

A full-length *DFR* cDNA was isolated from a trembling aspen cDNA library. Initial cloning of a *DFR* fragment and primary screening of a wounded aspen leaf cDNA library was performed by Trung Lam. Degenerate *DFR* nucleotide primers were designed from an amino acid sequence alignment of DFR enzymes from several plant species and PCR was performed with upstream primer DFR 2-D (5'-GAT/C CCN A/GAA/G AAT/C GAA/G A/GTN ATT/C/A AAA/G CC- 3') and downstream primer

DFR 3-U (5'-A/GAA A/GTA CAT CCA NG/CC NGT CAT C/TTT-3'). Aspen cDNA was used in PCR reactions with the DFR primers and a DNA product of predicted size (215 base pairs) was produced, which was extracted from agarose gels and cloned into a pBluescript T-Vector (Marchuk *et al.*, 1990). This resulting plasmid was named pDFR and a glycerol stock of *E. coli* containing pDFR was prepared. The pDFR plasmid was purified using the standard plasmid mini-prep protocol stated in Sambrook *et al.* (1989) and sequenced by the Core DNA sequencing facility (Medical Sciences Building, University of Alberta, Edmonton, Alberta) using the T3 and T7 primers. The *DFR* sequence was compared to other sequences in the Genbank database using the BLASTX program at the NCBI web page (www.ncbi.nlm.nih.gov), and found to be most similar to DFR enzymes from other plant species. Primary screening of a wounded aspen leaf Lambda ZAP II cDNA library (Stratagene, LaJolla, California) (Haruta *et al.*, 2001a) was performed using standard molecular techniques (Sambrook *et al.*, 1989). Thirty-three plaques that hybridized with the *DFR* fragment were picked and suspended in 500 µl of SM buffer containing 50 µl chloroform. Plaques were stored at 4 °C until analyzed.

*2.3.2. Screening of Aspen cDNA library and Isolation of DFR*

Bacteria from the pDFR glycerol stock were streaked onto LB agar plates (Sambrook *et al.*, 1989) containing 50 µg $mL^{-1}$ ampicillin, and incubated at 37 °C. Purified pDFR plasmid from a single colony was obtained using the QIAGEN Plasmid Midi Kit (Qiagen, Germany), following the protocol as stated in the QIAGEN Plasmid Purification Handbook (July 1999). The 215 base pair *DFR* fragment was isolated by restricting the pDFR plasmid with 25 U of *Xba*I and 25 U of *Xho*I in 1X React2 buffer

(Gibco BRL, Burlington, Ontario). The *DFR* fragment was electrophoresed through a 1.2% agarose gel and visualized on a transilluminator by staining with ethidium bromide. *DFR* was excised from an agarose gel, isolated using the QIAEX II gel purification kit, and used as a probe in the secondary screening of picked plaques from the primary screen of the wounded aspen leaf cDNA library.

All thirty-three phage solutions from the primary screen were tested in PCR reactions with the DFR primers to determine if *DFR* cDNA was present in the picked plaque. PCR reactions consisted of 3 U Taq polymerase, 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 100 μg $mL^{-1}$ BSA, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 50 μM of each dNTP, 50 nM of each primer and 0.5 μL of phage solution. DNA was visualized by staining with ethidium bromide, and analyzed under UV light using a MultiImage Light Cabinet (Alpha Innotech Corporation, San Leandro, California) and AlphaEase v. 5.1 software. Ten plaques from the primary screen that showed a PCR product of expected size were plated and titred according to standard procedures in Sambrook *et al.* (1989). XL1-Blue MRF' *E. coli* cells were grown to mid-log phase in 20 mL LB broth containing 0.2% maltose and 10 mM $MgSO_4$ for 4 h at 37 °C. Cells were pelleted by centrifugation, resuspended in 10 mM $MgSO_4$ and the concentration of the cell suspension was adjusted to an $OD_{600}$ of 0.5 with 10 mM $MgSO_4$. Dilutions of phage ($10^{-2}$, $10^{-4}$, $10^{-6}$) from plaques of the primary screen were incubated with an equal amount of the bacterial cell suspension and incubated for 15 min at 37 °C. Phage and cells were added to 6.5 mL NZY top agar that had been equilibrated to 48 °C, and plated on NZY agar plates. Plates were incubated at 37°C for 8 h. Titering revealed that plaques from the primary screen contained approximately 3.5 x $10^8$ p.f.u. $mL^{-1}$. For the secondary screen, the above

methods were followed using dilutions of $10^{-6}$ and $10^{-7}$ for each of the ten plaques from the primary screen.

Plaque lifts were performed using Hybond-N (Amersham, UK) nylon membrane filters for 2 min, and phage DNA was denatured and bound to the membranes by autoclaving for 2 min at 110 °C. Hybridization with the *DFR* probe was performed according to procedures in Sambrook *et al.* (1989). Briefly, membranes were placed in 6X SSPE solution for 30 min at room temperature, and prehybridized in 50 mL solution (5X SSPE, 50% formamide, 5X Denhardt's solution, 1% SDS, 100 µg denatured salmon sperm DNA) for 1 h at 42 °C. *DFR* probe was prepared by labeling the *DFR* fragment with $^{32}P$ using the T7 Quickprime kit (Pharmacia, Peapack, New Jersey) according to the manufacturer's protocol. The probe was purified and unincorporated nucleotides were removed by centrifugation through a Bio-spin 30 chromatography column (Bio-Rad, Hercules, California). Labeled *DFR* probe and the nylon filters were placed in 50 mL fresh prehybridization solution, and incubated for 16-20 h at 42°C on a shaker.

After the hybridization period, filters were washed twice in 5X SSPE, 0.5% SDS for 1 min at room temperature, and once in 1X SSPE, 1% SDS for 15 min at 62 °C. Eight of the ten plaques showed signals after exposure of nylon filters to Super RX medical X-ray film (Fuji, Tokyo, Japan), which was developed using a Kodak M35A X-OMAT processor, and these plaques were picked and suspended in 200 µL SM buffer containing 20 µL chloroform, and stored at 4 °C.

A tertiary screen was performed on the eight plaques using the methods stated above, and ten plaques that hybridized with the *DFR* probe were chosen for *in vivo* excision performed using the manufacturer's protocol (Stratagene, LaJolla, California).

Nine of the ten cDNA clones were successfully obtained by *in vivo* excision, and plasmid from each clone was isolated by the standard plasmid mini-prep protocol (Sambrook *et al.*, 1989) followed by precipitation in 6.5% PEG8000. Purified plasmid from each clone was sequenced on both strands using T3 (5'-AAT TAA CCC TCA CTA AAG GG-3') and T7 (5'-GTA ATA CGA CTC ACT ATA GGG C-3') primers and both the Thermosequenase dye terminator cycle sequencing kit (Amersham) with the Applied Biosystems 377 Automatic Sequencer, and the CEQ 2000 Dye Terminator Cycle Sequencing kit (Beckman) with the Beckman CEQ Automatic Sequencer. Analysis of the nucleotide sequences using the BLASTX program at the NCBI web page (www.ncbi.nlm.nih.org) revealed that five of the nine clones had high similarity to *DFR* from other plant species. One *DFR* clone, *DFR14a*, appeared to be a full-length clone as it had the expected 5' start codon, and was chosen for further sequencing.

### *2.3.3 Sequencing of full-length DFR cDNA*

Sequencing primers were designed based on initial and successive sequences of pDFR14a, and synthesized by the Molecular Biology Services Unit (Biological Sciences Building, University of Alberta, Edmonton, Alberta) and Gibco BRL custom primers (Canadian Life Technologies, Burlington, Ontario). A total of five sequencing primers were designed (see Results) and used so that the *DFR* cDNA was sequenced completely on both strands. DNA sequences were analyzed using Sequencher 4.1.2 (Gene Codes Corporation, Ann Arbor, Michigan) software. A glycerol stock of *E. coli* harbouring pDFR14a was prepared by freezing 0.85 mL of an overnight culture containing 0.15 mL of glycerol in liquid nitrogen, and the stock was stored at –80°C.

*2.4 Cloning and Sequencing of a Second DFR Sequence*

A second *DFR* sequence from aspen was cloned by a PCR-based approach. Two degenerate DFR primers were designed from conserved regions within the *DFR* gene from several plant species. These primers, which were designed to amplify all *DFR* genes that may be present in the aspen cDNA library, were as follows: DP-DFRS3 (5'-GTI A/CGI GCI ACI GTI A/CGI GAC/T CC-3') and DP-DFRA4 (5'-AA/GA/G TCA/G TCI AA/GA/G TGI ACA/G TA-3'). PCR amplification of aspen cDNA with these primers was performed using 34 cycles of 95 °C (30 sec)/ 48 °C (45 sec)/ 72 °C (60 sec). DNA products of expected size (600 base pairs) were extracted from agarose gels and cloned into pBluescript T-Vectors using standard ligation procedures as described in Sambrook *et al.* (1989). A 3:1 insert/vector ratio was used, and the appropriate amount of each DNA was incubated with T4 DNA ligase and 0.4 mM ATP for 12-16 h at 14 °C. Ligation mixtures were transformed into competent XL1-Blue MRF' *E. coli* cells by incubating the DNA and cells for 40 min on ice, and then immediately heating the mixture to 42 °C for 2 min. After heat shock, cells were grown in LB liquid medium for 1 h at 37 °C, and spread on LB agar plates containing 50 μg $mL^{-1}$ ampicillin, 30 μL of X-gal (60 mg $mL^{-1}$), and 30 μL of IPTG (60 mg $mL^{-1}$). Plates were incubated overnight for 14-16 h at 37 °C. White colonies were chosen for PCR with the DFR degenerate primers to confirm that the transformed plasmid contained the *DFR* insert. In order to differentiate between two different *DFR* alleles or genes, a primer was designed based on the substrate specificity region of *DFR* from pDFR14a (DP-DFRS4: 5'-GTG GAT GTT GAA GAA AAG AG-3'). This region is the most variable within the *DFR* gene when

comparing *DFR* genes from different plant species. Colonies containing the *DFR* insert were used in PCR with the specific DP-DFRS4 primer and the degenerate DP-DFRA4 primer. Of the 11 colonies containing the degenerate *DFR* insert, four did not have a PCR product when DP-DFRS4 and DP-DFRA4 were used as primers. These four colonies, and one colony that had a PCR product with the DP-DFRS4 and DP-DFRA4 primer pair, were grown in 1.5 mL LB medium for 16 h, and plasmid was purified by the standard plasmid mini-prep protocol (Sambrook *et al.*, 1989). Plasmid was used as template in sequencing as described above.

*2.5 Cloning and DNA Sequencing of Phenylpropanoid Gene Fragments*

Fragments of five phenylpropanoid gene cDNAs were isolated using a PCR-based approach. Primers for phenylalanine ammonia-lyase (*PAL*), 4-coumarate:CoA ligase (*4CL*), chalcone synthase (*CHS*), anthocyanidin synthase (*ANS*), and leucoanthocyanidin reductase (*LAR*) were designed from nucleotide or amino acid alignments of the respective phenylpropanoid genes from several plant species. Since *PAL* and *4CL* have been sequenced from *Populus* species, primers were designed based on the published sequences (Subramaniam *et al.*, 1993; Hu *et al.*, 1998). *PAL* was amplified using the PALS1 and PALA1 primers, and *4CL* was amplified using the 4CLS1 and 4CLA1 primers (Table 1). *CHS* and *ANS* primers were designed based on consensus sequence information from several plant species in public databases. *CHS* was amplified using the CHSS1 and CHSA1 primers, and *ANS* was amplified using the ANSS1and ANSA1 primers (Table 1). *LAR* primers were designed based on EST sequences in PopulusDB (http://poppel.fysbot.umu.se/) with the highest identity to the *Arabidopsis BAN* gene,

Table 1: The oligonucleotide primers used for cloning of phenylpropanoid gene fragments from aspen cDNA. The melting temperature ($T_m$) and the percentage of G+C nucleotides in the oligonucleotide (%GC) are listed.

| Oligo Name | Sequence (5'-3') | $T_m$ | %GC |
|---|---|---|---|
| PALS1 | GGC GTT ACT GGT TTT GGT GC | 62 °C | 55% |
| PALA1 | TTG AAG CCA TAA TCC AAA CTT GG | 62 °C | 40% |
| 4CLS1 | AGT GTG GCT CAA CAA/G GTA/T GAT GG | 69 °C | 50% |
| 4CLA1 | AAC/T GCA/G ACA GGA ACT TCA/T CC | 60 °C | 50% |
| CHSS1 | GCNATA/T/C AAA/G GAA/G TGGGGNCAA/G CC | 74 °C | 53% |
| CHSA1 | GGN CCN CCN GGA/G TGN GCA/T/G ATC C | 65 °C | 65% |
| ANSS1 | GAA/G TGGC/G AA/G GAC/T TAC/T TTC/T TTC/TC | 68 °C | 40% |
| ANSA1 | GGN GGC/T TCA/G CAA/G AAN CAN GCC | 65 °C | 57% |
| LARS1 | CAT GGC ATC CCA GTT GAC C | 60 °C | 58% |
| LARA1 | GTA CTC CTT GAA TTG CTG GC | 60 °C | 50% |

which encodes *LAR*. *LAR* was amplified using the LARS1 and LARA1 primers (Table 1).

Primers were tested with aspen cDNA using a gradient PCR thermocycler (Eppendorf, Germany) with an amplification program of 34 cycles of 95 °C (30 sec)/ 54 °C (45 sec)/ 72 °C (60 sec). The median annealing temperature was 55 °C and the gradient was ± 10 °C. Amplification of each phenylpropanoid fragment from 0.5 µL aspen cDNA was performed using 34 cycles of 95 °C (30 sec)/ 54 °C (45 sec)/ 72 °C (60 sec) in PCR reactions (see 2.3.2). DNA products were extracted from agarose gels and cloned into T-Vectors as described above. White colonies were selected for PCR screening using the appropriate primers and PCR amplification conditions described above. Colonies that contained plasmids with the appropriate insert were selected, and plasmid was purified using the QIAGEN plasmid midi kit (Qiagen, Germany). Plasmid was used in DNA sequencing with the T7 and T3 primers as described above. Sequences were analyzed using the BLASTX program at the NCBI web page (www.ncbi.nlm.nih.org) in order to confirm the identity of the gene fragment.

*2.6 Genomic DNA Extractions and Southern Hybridization*

Genomic DNA was isolated from trembling aspen using a genomic DNA extraction protocol (Doyle and Doyle, 1990) or a DNeasy Maxi Plant kit (Qiagen, Germany) and following the protocol in the DNeasy Plant Maxi handbook (April 1998). Genomic DNA extraction involved extracting 0.5 g of frozen aspen leaf tissue in 5 mL of 0.1M Tris-HCl (pH 8.0), 0.35 M sorbitol, 10% PEG8000 and 0.5% 2-mercaptoethanol, and centrifuging at 4000 r.p.m. for 10 min at 4 °C. The pellet was resuspended in 2.5 mL

0.1 M Tris-HCl (pH 8.0), 0.35 M sorbitol and 0.5% 2-mercaptoethanol, and incubated for 10 min at room temperature. Then, 2.8 mL of CTAB solution (2% CTAB, 0.1% Tris-HCl (pH 9.5), 20mM EDTA, 1.4M NaCl, 0.5% 2-mercaptoethanol) was added to the pellet suspension followed by a 10 min incubation at 65 °C. An equal volume of chloroform-isoamyl alcohol (24:1 v:v) was added and the mixture was centrifuged at 4000 r.p.m. for 10 min at room temperature. The aqueous layer was removed and DNA was precipitated by adding an equal volume of 100% isopropanol and incubating for 30 min at room temperature. DNA was collected by centrifugation at 6000 r.p.m. for 20 min at 4 °C, and resuspended in 10 mL wash solution (76% ethanol, 10 mM ammonium acetate). After a 20 min incubation at room temperature, genomic DNA was centrifuged at 3500 r.p.m. for 10 min at 4 °C, air-dried, and dissolved in 1 mL TE (10 mM Tris-HCl (pH 7.4), 1 mM EDTA). RNase A was added to a final concentration of 20 μg $mL^{-1}$, and the DNA was incubated for 30 min at 37 °C. The DNA-RNase mixture was extracted twice with equal volumes of phenol-chloroform (1:1 v:v), and genomic DNA in the aqueous layer was precipitated in 100% isopropanol for 10 min at room temperature. DNA was collected by centrifugation at 14,000 r.p.m. for 10 min at 4 °C, air-dried, and dissolved in 300 μL TE buffer. Genomic DNA was quantified by using a spectrophotometer to determine the absorbance at 260 nm, and quality was examined by electrophoresing through an agarose gel.

For Southern hybridization, genomic DNA (10 μg) was digested with 50 U of the restriction enzymes *Hin*dIII, *Nco*I, *Xba*I and *Eco*RI (Gibco/BRL), and separated on an agarose gel. Genomic DNA in agarose gels was depurinated (0.25 M HCl, 10 min), denatured (1.5 M NaCl, 0.5 M NaOH, 30 min), and neutralized (1.5 M NaCl, 1 M Tris-

HCl (pH 8.0), 30 min) in preparation for blotting in 10X SSPE to Zeta-Probe blotting membrane (Bio-Rad, Hercules, California) for 14-16 h. DNA was crosslinked to the membrane with 50 mJoule UV in a GS Gene linker UV chamber (Bio-Rad, Hercules, California). Southern hybridization was performed with the full-length $^{32}$P-labelled *DFR* using the same procedure as for the plaque lifts (see 2.3.2). Membrane filters were washed at 65 °C in 1X SSPE, 0.1% SDS for 30 min and 10 min (low stringency washes) and at 65 °C in 0.1X SSPE, 0.1% SDS for 30 min and 10 min (high stringency washes). Hybridization signals were determined by autoradiography or by exposure to a Phosphorimager screen (Molecular Dynamics, Sunnyvale, California).

*2.7 RNA Extraction and Northern Hybridization*

Total RNA was extracted from 0.5 g of aspen tissues according to the RNA extraction protocol of Chang *et al.* (1993) as modified by Haruta *et al.* (2001a). Frozen tissue was extracted with 5 mL CTAB buffer (2% w:v CTAB, 0.1 M Tris-HCl (pH 9.5), 0.02 M EDTA, 1.4 M NaCl, 2% v:v 2-mercaptoethanol) for 10 min at 65 °C, followed by extraction in 5 mL chloroform-isoamyl alcohol (24:1 v:v). The aqueous fraction was collected by centrifugation at 15,000 r.p.m. for 10 min at room temperature, and precipitated in three-fourths volume of 100% isopropanol for 10 min at room temperature. The nucleic acid was pelleted by centrifugation at 15,000 r.p.m. for 10 min at 4 °C, air-dried, and dissolved in 1 mL TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). The RNA was precipitated by adding 250 µL 10 M LiCl and incubating on ice for 1-2 h. RNA was collected by centrifugation at 15,000 r.p.m. for 10 min at 4 °C, and redissolved in 600 µL TE buffer. Following extraction in 1 volume of phenol-chloroform

(1:1 v:v), the RNA was precipitated in one-tenth volume 3 M sodium acetate (pH 4.5) and two volumes of 95% ethanol for 10 min at –80 °C. RNA was collected by centrifugation at 15,000 r.p.m. for 10 min at 4 °C, and resuspended in 300 µL TE buffer. RNA was quantified at 260 nm using a spectrophotometer, and quality was determined on agarose gels.

Northern analysis was performed by electrophoresing 15 µg of total RNA through 1.2% agarose-formaldehyde gels (1X MOPS, 6.66% formaldehyde (v:v)). RNA was visualized by staining with ethidium bromide. Gels were incubated in distilled water for 25 min to remove formaldehyde, and RNA was transferred to Zeta-probe blotting membrane (Bio-Rad, Hercules, California) in 10X SSPE for 16 h. After transfer, RNA was crosslinked to the membrane in a UV chamber. Northern hybridization was performed using various $^{32}$P-labelled probes in prehybridization solution (with 1.5 g dextran sulphate added) as described above. After hybridization, membranes were rinsed twice with 2X SSPE at room temperature, twice with 1X SSPE, 0.1% SDS for 30 min and 10 min at 65 °C, and, if required, twice with 0.1X SSPE, 0.1% SDS for 30 min and 10 min at 65 °C. Hybridization signals were visualized by autoradiography or by exposure to a Phosphorimager screen (Molecular Dynamics, Sunnyvale, California). For reprobing, blots were stripped of radioactive probe by washing in 0.1X SSPE, 0.5% SDS for 30 min at 95 °C.

*2.8 DFR Bacterial Overexpression*

The *DFR* full-length cDNA was expressed in *Escherichia coli* in order to determine if the expressed DFR protein had DFR enzyme activity. The coding sequence

of *DFR* was PCR-amplified from the full-length *DFR* plasmid clone (pDFR14a) using the upstream primer DFRUPPER (5'- GGG CGG ATC CAA GAT GGG AAC AGA AGC -3') and the downstream primer DFRLOWER (5'- GGG GAA GCT TGA GGA ACA ATC AGG ACG -3'). The fragment was restricted with *Bam*HI and *Hin*dIII (sites located within the primer sequences) and subcloned into the *Bam*HI and *Hin*dIII restriction sites of the pQE30 bacterial expression vector (Qiagen, Germany). The plasmid was transformed by the heat shock method into *E. coli* strain M15 (pREP4) and grown on 50 $\mu g\ mL^{-1}$ ampicillin-LB plates for 16 h at 37 °C. Colonies containing the *DFR* insert within the expression vector were selected by PCR screening with the DP-DFRS1 and DP-DFRA3 primers, and identifying PCR products on agarose gels. Colonies containing the *DFR* overexpression vector were grown in 3 mL LB medium containing 50 $\mu g\ mL^{-1}$ ampicillin and 1mM IPTG for 16-20 h at 30 °C. Bacteria was pelleted by centrifugation, and resuspended in 0.1 M Tris-HCl (pH 7.4) containing 20 mM sodium ascorbate. Cells were disrupted by sonication using a Sonic Dismembrator Model 300 (Fisher) with a micro-tip operating at 35%. The suspension was centrifuged, and the supernatant directly used in the DFR enzyme activity assay as described below.

*2.9 DFR Plant Transformation Vector Construction*

Plant transformation vectors expressing full-length *DFR* in both the sense and antisense orientations were constructed for future *Agrobacterium*-mediated transformation. Sense constructs were manufactured by restricting the pDFR14a plasmid with 20 U *Xba*I and 20 U *Kpn*I, and ligating the agarose gel-extracted full-length *DFR* coding sequence into the *Xba*I and *Kpn*I sites of the pGEMCaNOS plant transformation

vector (obtained from Dr. M. Christopher). In this vector, *DFR* is flanked by a duplicated 35S cauliflower mosaic virus (CaMV) constitutive promoter and *nos* terminator sequences. The vector was transformed into XL1-Blue MRF' competent *E. coli* cells, and plasmid was purified using a QIAprep Spin Miniprep kit (Qiagen, Germany) following the protocol in the QIAprep Miniprep handbook (July 1999). Sense orientation was confirmed by sequencing the junctions as described above using primers designed for the 3' end of the 35S CaMV promoter (35S primer) and 5' end of the *nos* terminator (nos primer).

Antisense constructs were manufactured by PCR-amplifying the full-length *DFR* sequence from pDFR14a using the DFRUPPER and DFRLOWER2 (5'-GGG CGG ATC CGA GGA ACA ATC AGG ACG-3') primers. PCR-generated fragments were extracted from agarose gels and restricted with 20 U *Bam*HI (restriction sites located within the primer sequences). The *Bam*HI-digested *DFR* fragment was incubated with *Bam*HI-digested pGEMCaNOS in a ligation reaction (as described above), and transformed into XL1-Blue MRF' competent *E. coli* cells by the heat shock method. Cells growing on LB plates containing 50 μg $mL^{-1}$ ampicillin were screened by PCR with DP-DFRS1 and DP-DFRA3 to select colonies containing the *DFR* insert. To determine the orientation of the *DFR* insert, five colonies were screened with four different combinations of PCR primers: (1) 35S and DP-DFRS1, (2) nos and DP-DFRS1, (3) 35S and DP-DFRA3, and (4) nos and DP-DFRA3. Three colonies that displayed PCR products on agarose gels from primer combinations (1) and (4) had the *DFR* insert in the desired antisense orientation. Additionally, the antisense orientation of *DFR* was confirmed by sequencing the junctions as described above using the 35S and nos primers.

*2.10 Biochemical Assays*

*2.10.1 Condensed Tannin Assays*

Condensed tannin (proanthocyanidin) concentrations were determined using the anthocyanidin method described by Porter *et al.* (1986). Condensed tannins were extracted from 50 mg of aspen leaf tissue with 10 mL of acetone-water (70:30 v:v) for either 2 h or overnight at room temperature on a shaker. The extraction of condensed tannins from aspen tissue was found to be optimal in 70 % acetone, as all condensed tannins were extracted from aspen leaf tissue after 30 min. Acetone was found to be a more efficient extraction solvent than methanol, as numerous extractions of the same tissue were required to extract all condensed tannins with 80% methanol. Two mL of proanthocyanidin extract was added to eight mL of *n*-butanol-hydrochloric acid (95:5 v:v) and 0.267 mL of ferric reagent (2% solution (w:v) of ferric ammonium sulphate dodecahydrate in 2M hydrochloric acid). The solution was heated to 95 °C in a waterbath for 40 min, cooled for 30 min at room temperature, and the absorbance recorded at 550 nm using a spectrophotometer. Purified sainfoin (*Onobrychis viciifolia*) tannin obtained from Dr. M. Gruber (Agriculture and Agri-Food Canada, Saskatoon, Saskatchewan) was used as a standard.

Tannin in aspen leaf extracts was also assayed by the radial diffusion method described by Hagerman (1987). One mL of proanthocyanidin extract was evaporated under vacuum into a small volume (approximately 50 µl). Successive 8 µL aliquots were added to a 4.0-mm well in a 1% (w:v) agarose-BSA plate (50 mM acetic acid, 60 µM

ascorbic acid (pH 5.0), 0.1% (w:v) BSA). Agarose-BSA plates consisted of 10 mL agarose-BSA solution solidified in a 150 mm petri dish. Plates were incubated at 30 °C for 96 h, and diameters of the precipitation rings measured using a Mecanic type 6911 caliper (Manostat Corporation, Switzerland). Purified sainfoin tannin was used as a standard.

*2.10.2 Anthocyanin Assay*

Anthocyanins were extracted from aspen leaf tissue (100 mg) using 10 mL of 1% hydrochloric acid in methanol for 24 h at 4 °C (Strack and Wray, 1989). The extracts were clarified by centrifugation, and an estimate of the total anthocyanin content of the samples was determined by absorbance measurements at 530 nm (absorption peak of anthocyanin) and 657 nm (absorbance of chlorophyll in solvent extract) using a spectrophotometer. The final absorbance was determined by the formula $A_{final} = A_{530} - 0.25 \times A_{657}$, which corrected for the presence of degradation products of chlorophyll in the acidic methanol solution that contributed to the absorbance at 530 nm (Mancinelli and Schwartz, 1984).

*2.11 DFR Enzyme Activity Assay*

DFR activity was measured using the assay described by Stafford and Lester (1982). Approximately 1.5 g of frozen aspen leaf tissue was extracted in 5 mL of 0.1 M borate-hydrochloric acid (pH 8.8) buffer containing 20 mM sodium ascorbate, 10% (v:v) glycerol, 0.3 g dry polyvinylpolypyrrolidone (Sigma), and 0.5 g dry Amberlite XAD-4 ion-exchange resin (Sigma). Samples were centrifuged and the supernatant fraction

desalted through a PD-10 desalting column (Pharmacia) containing Sephadex G-25 medium resin equilibrated with 0.1 M Tris-hydrochloric acid (pH 7.4) and 20 mM sodium ascorbate. Protein concentrations were determined by the Bradford method (Bradford, 1976) using BSA as a standard.

To measure DFR activity, a 1-ml reaction tube was prepared that contained 0.3 ml enzyme extract, 0.1 M Tris-HCl (pH 7.4) buffer, 1 mM dihydroquercetin (±-taxifolin; Sigma) as substrate, and 1 mM β-NADPH (Sigma) as reducing agent. A NADPH-regenerating system consisting of 1 U glucose-6-phosphate dehydrogenase and 6 μM glucose-6-phosphate was also added to the reaction mixture. Reactions were incubated at 30 °C for 30 min, and stopped by the addition of 6 N hydrochloric acid. Leucoanthocyanidin product was extracted with ethyl acetate, and the ethyl acetate was evaporated under vacuum. One mL of *n*-butanol:hydrochloric acid (95:5 v:v) was added and the solution was heated for 40 min at 95 °C to convert the leucoanthocyanidin to anthocyanidin, which was measured by absorbance at 550 nm using a spectrophotometer. The absorbance was converted into nmol cyanidin using the molar extinction coefficient 34 700 (Stafford and Lester, 1981).

## 3. Results

### *3.1 Isolation and Sequencing of Full-length DFR from Trembling Aspen*

DFR is a key enzyme involved in the synthesis of condensed tannins and the isolation of a full-length *DFR* cDNA would provide an important tool for analyzing induction of condensed tannin synthesis at the molecular level. A wounded leaf cDNA library from aspen (Haruta *et al.*, 2001a) was initially screened with a previously isolated PCR-generated *DFR* probe (T. Lam and P. Constabel, unpublished data). Thirty-three positive plaques were suspended in SM buffer, and stored at 4 °C. These phage suspensions were screened by PCR with DFR primers 2D and 3U in order to determine which suspensions contained *DFR* clones. *DFR* products were obtained from 14 of the phage suspensions, and 10 were chosen for further screening. Secondary screening was initially unsuccessful as the $^{32}$P-labeled *DFR* probe was washed from the filters using the wash procedure of twice in 5X SSPE, 0.5% SDS for 1 min at room temperature, and twice in 1X SSPE, 1% SDS for 30 min at 65°C. Less stringent wash procedures (twice in 5X SSPE, 0.5% SDS for 1 min at room temperature, once in 1X SSPE, 1% SDS for 15 min at 62 °C) were used after reprobing the filters with $^{32}$P-labeled *DFR*, and a total of 13 plaques were picked from the eight phage suspensions that showed *DFR* signals. Phage from these 13 plaques was rescreened (tertiary screen) and nine positive clones were successfully excised and sequenced using the T3 and T7 primers. Comparison to sequences in Genbank using the BLAST search tool revealed that five of the sequenced clones had high sequence similarity to previously isolated *DFR* genes from other plant species. One cDNA clone, *DFR14a*, was completely sequenced on both strands, and found to encode the full-length protein coding sequence of aspen DFR. The

oligonucleotide primers used to sequence the aspen *DFR* cDNA are listed in Table 2, and the primers are shown arranged on the aspen *DFR* nucleotide sequence in Figure 2. This cDNA was named *PtDFR*.

The nucleotide sequence of *PtDFR* was found to be 1041 bp in length with an open reading frame that is predicted to encode a protein of 346 amino acid residues. The predicted molecular mass of aspen DFR is 38.8 kD (Swiss-Prot, ExPASy, http://ca.expasy.org). The nucleotide sequence and predicted amino acid sequence were compared with other proteins in Genbank, EMBL, and SWISSPROT, and were shown to have a high sequence identity with *DFR* genes previously cloned from other plant species (Figure 3). Aspen DFR showed the highest identity with DFR proteins from *Vitis vinifera* (76.0%), *Malus x domestica* (74.0%), *Rosa hybrida* (72.0%), *Fragaria x ananassa* (71.6%), and *Camellia sinensis* (71.4%). Sequence analysis of aspen DFR indicates that the conserved, N-terminal putative NADP-binding domain is present (Lacombe *et al.*, 1997). Computational analysis using PSORT (http://psort.nibb.ac.jp) revealed that PtDFR is not predicted to have an N-terminal signal sequence, though PtDFR may be peripherally associated with membranes, based on hydrophobic areas within the PtDFR amino acid sequence. Interestingly, the 134$^{th}$ residue in the substrate specificity region, asn in most plant DFR genes, is an asp residue in aspen. This residue is proposed to be important in substrate specificity (Johnson *et al.*, 2001) (see Discussion).

Many plant species contain multiple copies of genes involved in the phenylpropanoid pathway. Multiple *DFR* genes have been found in a number of plants

Table 2: The oligonucleotide primers designed for sequencing of the full-length aspen DFR cDNA (*PtDFR*). The melting temperatures ($T_m$) and percentage of G+C nucleotides in the oligonucleotide (%GC) are listed.

| Oligo Name | Sequence (5'-3') | $T_m$ | %GC |
|---|---|---|---|
| DP-DFRS1 | ACT GGA CAT CAT GAG AGC | 43 °C | 50% |
| DP-DFRA1 | GAT TTG CCA CAT GCT CTC | 43 °C | 50% |
| DP-DFRS2 | GCT AAC ATT CAC GAT CTG C | 44 °C | 47% |
| DP-DFRA2 | GAG GAC ACA ATC AGG ACG | 45 °C | 55% |
| DP-DFRA3 | GCA GAT CGT GAA TGT TAG C | 44 °C | 47% |

Figure 2: Nucleotide sequence of the full-length *DFR* (*PtDFR*) from aspen. The oligonucleotide primers used to sequence *PtDFR* are shown in black highlight. Overlap between oligonucleotide primers is shown in grey highlight.

ATTCGGCACGAGCTTACCAAGCCAGTCTAGATCAGAAACACCAGACATTGCTTTCAACT

TCCTACCTGACCCGAGTTCTCTCCACCATCTGTCTGAGAATTAAAG**ATG**GGAACAGAAG

CTGAAACAGTGTGTGTGACAGGTGCATCAGGCTTCATCGGATCATGGCTAATTATGCGA

CTACTAGAGAAAGGTTATGCTGTCCGAGCCACCGTCCGAGACCCTGATAACATGAAGAA

GGTGAAGCATTTGCTGGAACTGCCAAAGGCATCAACCCACTTGACTTTATGGAAAGCTG

ATCTTTCTGAAGAGGGGAGTTATGATGAAGCAATTCAAGGGTGCACAGGAGTGTTCCAT

GTTGCCACTCCCATGGATTTTGAATCCAAGGACCCCGAGAACGAGGTGATCAAGCCAAC

**DP-DFRS1 ->  <- DP-DFRA1**

AATCAACGGAGT[redacted]AAAAACAATCAGAAAGA

TAATATTCACGTCATCTGCAGGAACTGTGGATGTTGAAGAAAAGAGAAAACCAGTGTAC

GATGAAAGCTGCTGGAGTGATTTGGACTTTGTTCAGGGTATAAAAATGACTGGATGGAT

GTATTTCGTGTCCAAGACTTTAGCAGAGCAAGCTGCGTGGAAGTTTGCTGAAGATAATA

ACCTGGATTTTATCAGCATCATACCAACTCTTGTTGTTGGCCCATTTATCATGCAATCA

ATGCCACCAAGTCTCCTGACAGCGCTTTCACTGATCACTGGAAATGAAGCTCATTATGG

GATTTTAAAGCAGGGACATTATGTGCACTTGGATGACCTCTGCATGTCTCACATCTTTT

**DP-DFRS2 ->**

TGTACGAGAATCCAAAAGCAGAGGGCCGCTACATCTGCAACTCGGATGAT[redacted]

**<- DP-DFRA3**

[redacted]CCAATTACTCAGAGAAAAATACCCAGAATACAATGTCCCTGATAAGTT

CAAAGATATTGATGAGAATCTGGCGTGTGTTGCTTTCTCATCCAAAAAGCTGACGGACT

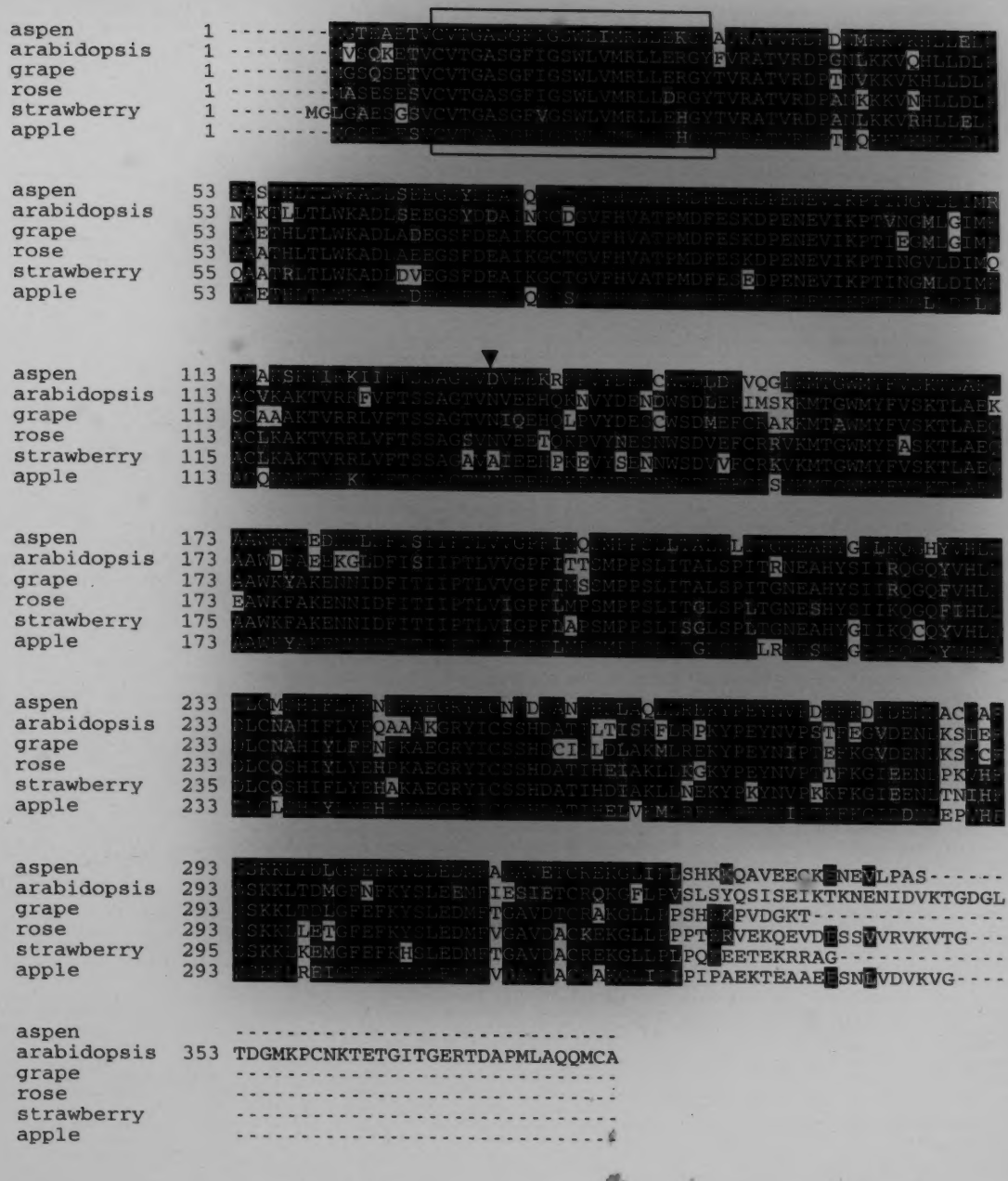
TGGGTTTTGAGTTCAAATACAGCTTGGAGGACATGTTCGCAGGTGCTGTGGAAACATGC

AGAGAAAAGGGACTAATTCCCCTTTCTCATAAGAAACAAGCCGTCGAGGAATGCAAAGA

**<- DP-DFRA2**

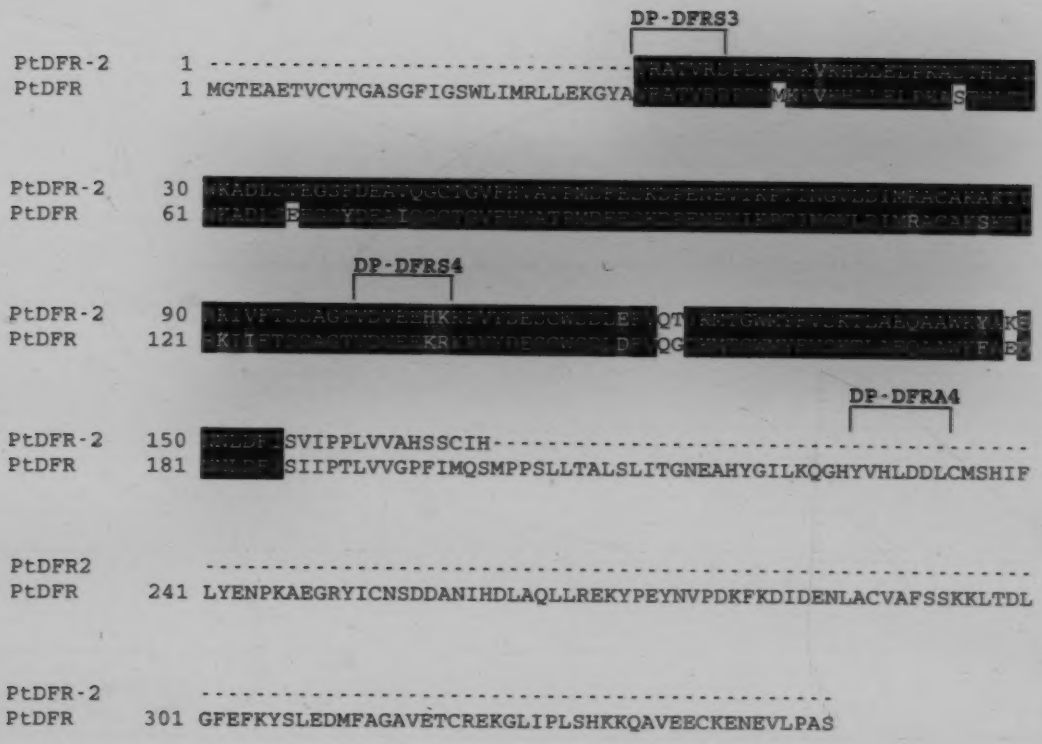
AAATGAAGTGCTCCCTG[redacted]TGA[redacted]GGTCCTGTTATTCCA

Figure 3: Multiple sequence alignment of the predicted PtDFR amino acid sequence with other DFR sequences. Identical amino acids are shown in black (80%); similar amino acids are shown in grey. Accession numbers are AF117268 for apple (*Malus* x *domestica*), Y11749 for grape (*Vitis vinifera*), D85102 for rose (*Rosa hybrida*), AF02968 for strawberry (*Fragaria* x *ananassa*), and AB007647 for *Arabidopsis thaliana*. Alignments were performed using the ClustalW 1.8 multi-alignment tool (searchlauncher.bcm.tmc.edu) and Boxshade (www.ch.embnet.org/software/BOX_form.html). The triangle indicates the asp residue thought to be important for substrate specificity, and the conserved NADPH-binding region is boxed.


aspen
arabidopsis
grape
rose
strawberry
apple

(Bernhardt *et al.*, 1998; Beld *et al.*, 1989; Inagaki *et al.*, 1999; Moyano *et al.*, 1998; Helariutta *et al.*, 1993). A PCR strategy was used to attempt to clone a second *DFR* from aspen, which could be used to determine whether there is differential regulation of different members of a *DFR* gene family. Degenerate primers were designed based on highly conserved sequences in DFR from many plant species (see Figure 3). The wounded leaf aspen cDNA library (Haruta *et al.*, 2001a) was screened with these primers in a PCR reaction, and DFR products of approximately 600 bp were cloned into T-vector plasmids. Eleven DFR clones were screened with a specific upstream primer based on the substrate specificity region of PtDFR, and the downstream DFR degenerate primer. Four of the DFR clones did not display PCR products using these primers and were predicted to be *DFR* sequences different than *PtDFR*. These four clones were sequenced, and comparison with sequences in Genbank using the BLAST search tool revealed that partial sequences from all four *DFR* clones showed the highest sequence identity to *DFR* nucleotide sequences from other plant species. Furthermore, all four clones (*PtDFR-2*) were identical, and showed an 89% sequence identity to the *PtDFR* sequence. However, there were a number of nucleotide differences between the *PtDFR-2* genes and the *PtDFR* nucleotide sequence that were predicted to result in different amino acids in the PtDFR-2 protein (Figure 4). Of the 18 amino acids that were different between PtDFR and PtDFR-2, 13 of these differences were to amino acids that had a similar polarity in their side chains. Additionally, nucleotide differences were predicted to result in three different amino acids in the substrate specificity region of PtDFR-2 compared to PtDFR. All of these amino acid differences were similar to amino acids (K138H, R139K, D152E)

Figure 4: Sequence alignment of the predicted PtDFR and PtDFR-2 amino acid sequences. Identical amino acids are shown in black; similar amino acids are shown in grey. Alignments were performed using the ClustalW 1.8 multi-alignment tool (searchlauncher.bcm.tmc.edu) and Boxshade (www.ch.embnet.org/software/BOX_form.html). The locations of the degenerate oligonucleotide primers (DP-DFRS3 and DP-DFRA4), and the specific *PtDFR* primer based on the proposed substrate specificity region (DP-DFRS4) are shown.


DP-DFRS3
PtDFR-2 1
PtDFR 1 MGTEAETVCVTGASGFIGSWLIMRLLEKGYA
PtDFR-2 30
PtDFR 61
DP-DFRS4
PtDFR-2 90
PtDFR 121
DP-DFRA4
PtDFR-2 150
PtDFR 181 SIIPTLVVGPFIMQSMPPSLLTALSLITGNEAHYGILKQGHYVHLDDLCMSHIF
PtDFR2
PtDFR 241 LYENPKAEGRYICNSDDANIHDLAQLLREKYPEYNVPDKFKDIDENLACVAFSSKKLTDL
PtDFR-2
PtDFR 301 GFEFKYSLEDMFAGAVETCREKGLIPLSHKKQAVEECKENEVLPAS

with side chains of the same polarity. Site-directed mutagenesis of the amino acids at these three positions did not result in a DFR enzyme with altered substrate preferences (Johnson *et al.*, 2002). Thus, these differences in the *PtDFR-2* sequence probably do not result in the synthesis of a second DFR enzyme with a different substrate specificity compared to PtDFR (see Discussion).

In order to amplify the 3' end of *PtDFR-2*, a sense primer based on the substrate specificity region of PtDFR-2 (DFR-2; 5'-GAT TTG GAA TTT GTC CGG ACC-3') was made, and used in PCR reactions with aspen RNA and the N-LOCK primer (5'-$[T]_{20}$N-3'), which was designed to bind to the 3'-polyadenylation sequence on aspen mRNA. Multiple PCR reactions were unsuccessful and additional *PtDFR-2* sequence information was not gained. Phenylpropanoid genes that are present as gene families in other plant species usually have a sequence identity between 60-70% (Hu *et al.*, 1998; Lurin and Jouanin, 1995). Since the partial nucleotide sequence of *PtDFR-2* is 89% identical to the *PtDFR* sequence, the two *DFR* sequences in aspen are most likely alleles of the *DFR* gene. This is supported by Southern blot analysis (see 3.3) (see Discussion).

*3.2 Bacterial Expression of DFR and Enzyme Assay*

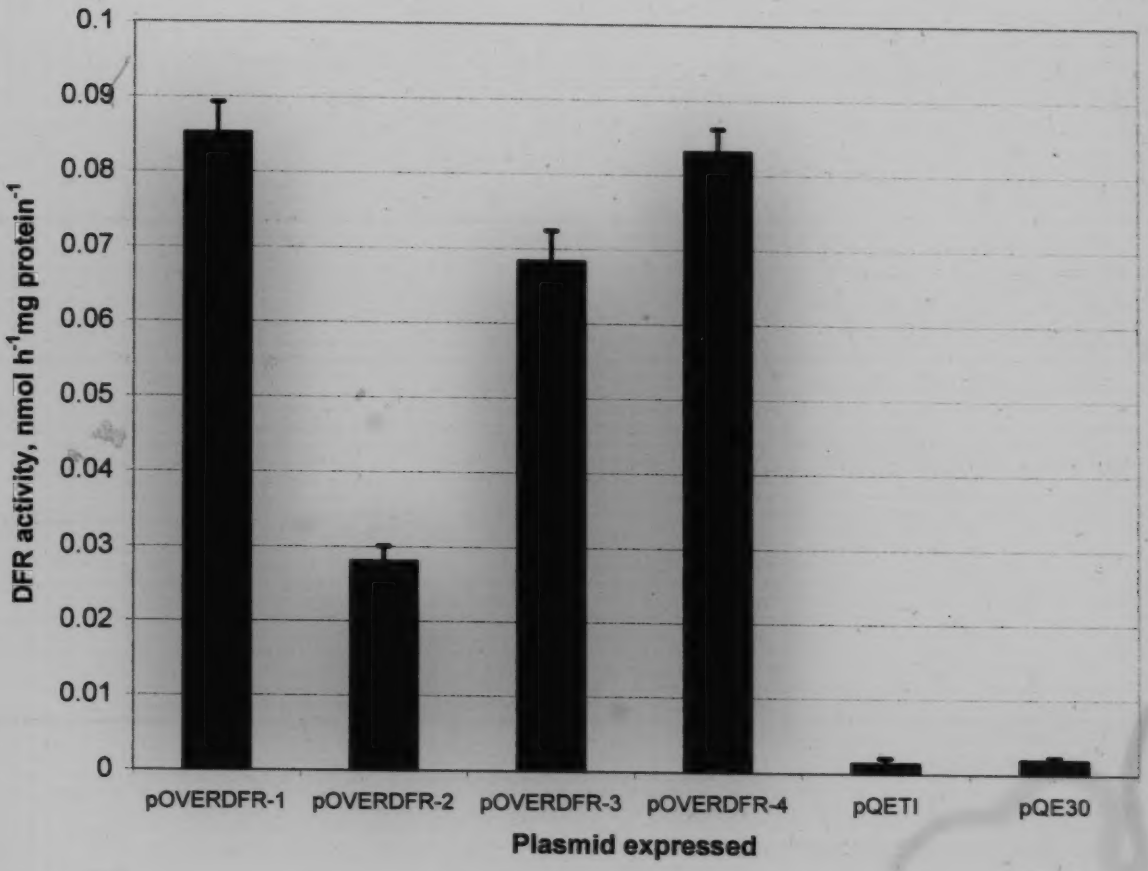
In order to show that the *PtDFR* cDNA encoded a functional DFR enzyme, *PtDFR* was expressed in *Escherichia coli*. *PtDFR* was PCR amplified from the pDFR14a plasmid using the DFRUPPER and DFRLOWER primers, which added a *Bam*HI (5') and a *Hin*dIII (3') restriction site to the ends of the *PtDFR* sequence. The procedure of directionally cloning the full-length *DFR* into the *Bam*HI and *Hin*dIII sites

of the pQE30 bacterial expression vector (Qiagen, Germany) was optimized after problems with obtaining viable competent cells, and troubleshooting the restriction of the PCR-generated *PtDFR* fragment. The restriction enzyme *Hin*dIII does not restrict DNA efficiently when the recognized restriction site is near the end of a DNA sequence (New England Biolabs, 1995). The PCR-generated *PtDFR* sequence was successfully restricted by first incubating the DNA with 30 units of *Hin*dIII in buffer containing 50mM NaCl for 1.5 h at 37 °C, and then adding 30 units of *Bam*HI, adjusting the NaCl concentration to 100mM, and incubating the reaction for 12-16 h at 37 °C. The restricted *PtDFR* sequence was successfully cloned into the pQE30 bacterial overexpression vector, and the *DFR* expression plasmid was transformed into M15 (pREP4) strain of *E. coli*. Cultures containing the *DFR* expression vector were induced with IPTG, sonicated and the supernatant was used in DFR activity assays. All *E. coli* colonies tested that harbored the *PtDFR* cDNA displayed DFR activity in the range of 0.03 – 0.09 nmol substrate reduced $h^{-1}$ mg $protein^{-1}$ (Figure 5). Control cultures containing only the pQE30 bacterial expression vector or a construct expressing trypsin inhibitor showed low DFR activity (0.001 nmol substrate reduced $h^{-1}$ mg $protein^{-1}$). Thus, *PtDFR* encodes a functional DFR enzyme.

*3.3 Southern Analysis of DFR in Aspen*

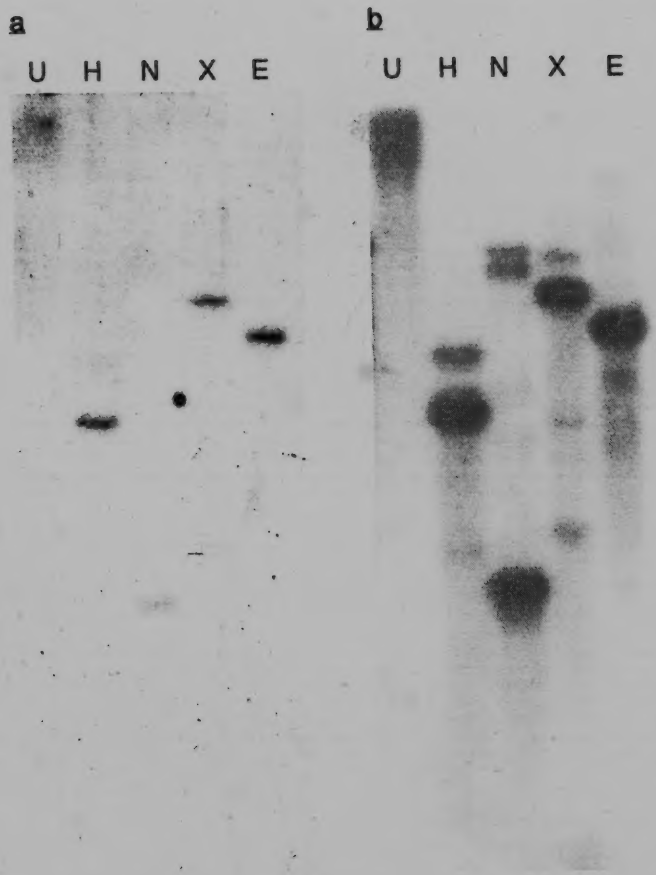
Southern analysis was performed in order to estimate the number of copies of *DFR* within the aspen genome. Genomic DNA from two different aspen genotypes, A2 and C1, was extracted using a genomic DNA extraction protocol (Doyle and Doyle, 1990). Analysis on agarose gels revealed that genomic DNA from the C1 genotype was

Figure 5. Measurement of DFR activity in protein extracts from *E. coli* expressing the *PtDFR* expression vector. Protein was extracted, desalted, and assayed for DFR activity as described in Materials and Methods. The pOVERDFR plasmids expressed the *PtDFR* sequence; pQETI is a plasmid expressing trypsin inhibitor; pQE30 is the bacterial expression vector. Each value is the mean of 3 colonies assayed; bars represent the standard error.


0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
DFR activity, nmol h-1mg protein-1
pOVERDFR-1
pOVERDFR-2
pOVERDFR-3
pOVERDFR-4
pQETI
pQE30
Plasmid expressed

of high molecular weight and high quantity (150 µg), but DNA from A2 was degraded. For the isolation of A2 genomic DNA, a DNeasy Maxi Plant kit (Qiagen, Germany) was used. Analysis on agarose gels revealed that A2 genomic DNA isolated with the DNeasy Maxi Plant kit was intact, but was present in a low yield (60 µg). C1 and A2 genomic DNA (1 µg) was separately digested with 20 U of *Hin*dIII, *Nco*I, *Xba*I, *Eco*RI, *Pst*I, *Kpn*I and *Bam*HI to test for adequate restriction by each enzyme. The restriction enzymes *Hin*dIII, *Nco*I, *Xba*I and *Eco*RI digested the A2 and C1 genomic DNA sufficiently as there were no high molecular weight bands appearing in the agarose gel. Ten µg of A2 and C1 genomic DNA was digested with 50 U each of *Hin*dIII, *Nco*I, *Xba*I and *Eco*RI for Southern analysis. Restricted DNA was blotted and hybridized with the full-length *PtDFR* probe. One hybridizing band was detected on Southern blots washed at high stringency (Figure 6a). This suggests that aspen contains a single *DFR* gene. Low stringency washes revealed the presence of three or four minor bands that hybridized with the *PtDFR* probe (Figure 6b) indicating that there may be other *DFR*-like genes within the aspen genome as revealed by the presence of multiple bands at low stringency. The enzyme immediately downstream of DFR in condensed tannin synthesis (LAR) is encoded by a *DFR*-like gene (the *BAN* gene in *Arabidopsis*) (Devic *et al.*, 1999), and the presence of multiple bands on Southern blots washed at low stringency may represent *BAN* homologues within the aspen genome (see Discussion).

Figure 6. Southern blot analysis of *PtDFR* in aspen (clone A2). Genomic DNA was restricted, electrophoresed, and hybridized with the *PtDFR* cDNA as described in the Materials and Methods. 6a and 6b show the same blot washed at high and low stringency, respectively. H = *Hin*dIII; N = *Nco*I; X = *Xba*I; E = *Eco*RI; U = uncut DNA.


a
U H N X E
b
U H N X E

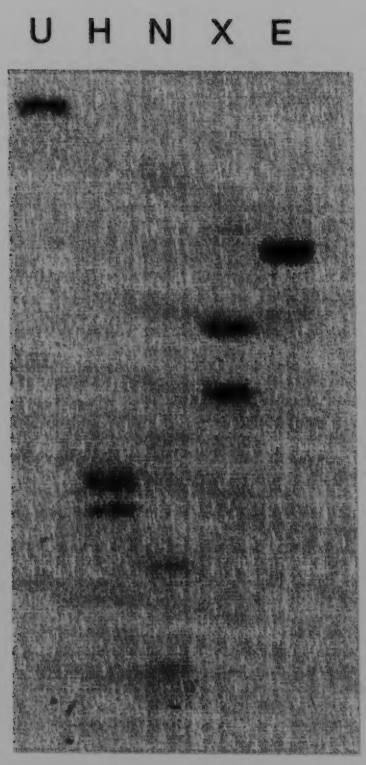
The results of Southern analysis suggests that *PtDFR-2*, which has an 89% sequence identity to *PtDFR* at the nucleotide level, is probably a second allele rather than a second *DFR* gene. Only one hybridizing *DFR* band was detected on Southern blots washed at high stringency, and since *PtDFR* and *PtDFR-2* are very similar, the *PtDFR* probe is expected to cross-hybridize with both sequences on the Southern blots. The pattern of bands detected with the *PtDFR* probe was different between two different aspen genotypes as revealed by Southern blot analysis. The hybridization patterns of *PtDFR* in the C1 and A2 genotypes were different, though only one band appeared on Southern blots washed at high stringency for both genotypes (data not shown).

For comparison, Southern blotting was also conducted with hybrid poplar (*Populus trichocarpa x Populus deltoides*) genomic DNA. Hybrid poplar DNA was extracted by the genomic DNA extraction procedure (Doyle and Doyle, 1990) and used in Southern analysis. Hybridization with the *PtDFR* probe revealed two hybridizing signals at high stringency conditions (Figure 7). This result is expected as a different *DFR* allele is contributed to the hybrid poplar genome by each of the parental species. Thus, it appears that poplars have one copy of the *DFR* gene in their genomes.

### *3.4 Wound-Induced Expression of DFR in Aspen*

Previous studies have demonstrated that concentrations of condensed tannins in leaves are variable between different aspen genotypes, and within aspen trees of the same genotype (Hwang and Lindroth, 1997). The Constabel lab has successfully

Figure 7. Southern blot analysis of *DFR* in hybrid poplar (*P. trichocarpa* x *P. deltoides*). Genomic DNA was restricted, electrophoresed, and hybridized with the *PtDFR* cDNA as described in the Materials and Methods. The blot was washed at high stringency. H = *Hin*dIII; N = *Nco*I; X = *Xba*I; E = *Eco*RI; U = uncut DNA.


U H N X E

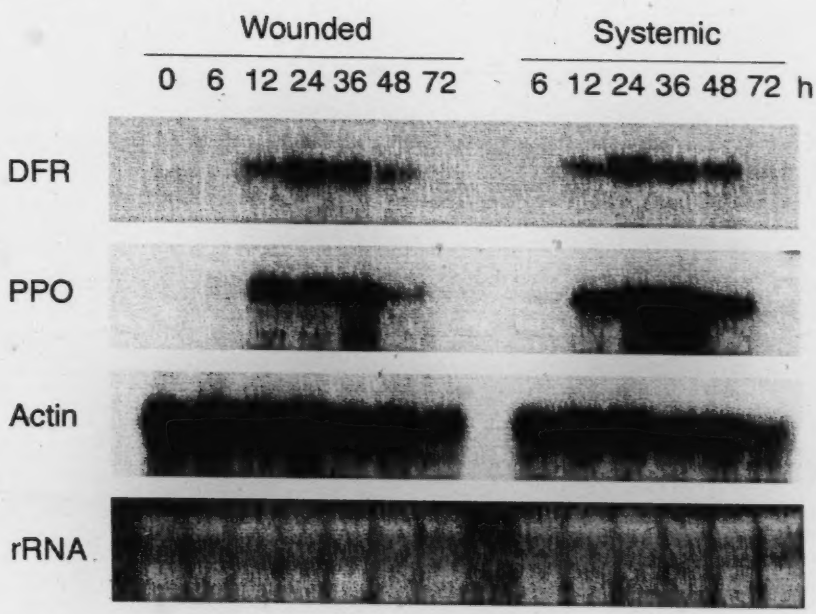
micropropagated four different aspen genotypes (clones), and leaf tissue from all four of these aspen genotypes was used in numerous *n*-butanol:HCl assays to determine condensed tannin concentrations so that the clone with the least variation in foliar condensed tannins could be used in subsequent experiments. All genotypes displayed variable concentrations of condensed tannins in leaf tissue; between 0.3-33 mg $g^{-1}$ fresh weight when grown in the University of Alberta's Biotron. Furthermore, individual trees of the same genotype also differed widely in concentrations of foliar condensed tannins. Genotypes C1 and C4, which were chosen for propagation from the field due to these clones showing high and low concentrations of foliar condensed tannins respectively, did not display the same differences in condensed tannin concentration as observed in the field. This difference may be due to low light conditions in the growth chamber compared to high light conditions in the field, as light has been shown to affect condensed tannin accumulation (Hemming and Lindroth, 1999). Thus, preliminary experiments did not lead to the choice of one clone among the four aspen clones that displayed the least variability in foliar concentrations of condensed tannins. However, for most experiments, clones A2 and G2 were used as these clones survived the best and displayed good health using the propagation techniques and growth chamber conditions described.

Since condensed tannin concentrations were variable between clones and within clones, leaf series experiments were performed in order to determine which leaves had the least variability in condensed tannin concentration. Multiple experiments that involved measuring condensed tannin concentrations in leaves of different ages suggested that the least variation in condensed tannins was present in leaves of LPI 9-21 in aspen

trees grown in the Biotron growth chamber. Thus, leaves of LPI 12-19 were used in experiments for molecular and biochemical analysis of the effects of wounding on condensed tannin synthesis.

Previous experiments have revealed that aspen has a strong inducible defense response (Haruta *et al.*, 2001a; Haruta *et al.*, 2001b). In these experiments, wound induction of defense genes was shown to be dependent on leaf age in aspen, where defense genes from young leaves displayed stronger wound induction compared to old leaves. The expression of *PtDFR* in response to mechanical wounding was analyzed using the *PtDFR* cDNA as a probe in northern analysis. Wound time course experiments were conducted by crushing the edges of leaves of LPI 16-19 on three-month old aspen plants with a hemostat to mimic herbivore damage. Both wounded leaves and unwounded (systemic) leaves (LPI 12-15) were harvested at appropriate times after wounding, and RNA was extracted and analyzed by northern hybridization. Mechanical wounding induced *PtDFR* expression to high levels in both wounded and systemic leaves (Figure 8). *PtDFR* mRNA expression was also detected consistently at low levels in control (0 h) leaves. Induced expression of *PtDFR* was detectable after 12 h, peaked after 24 h in both the wounded and systemic leaves, and declined to constitutive levels after 72 h. For comparison, the blot was stripped and rehybridized with a full-length probe encoding polyphenol oxidase (*PPO*) from aspen, a known wound-inducible gene in both poplar and aspen (Constabel *et al.*, 2000, Haruta *et al.*, 2001a). The kinetics of *PPO* mRNA induction were almost identical to those for *PtDFR*, although *PPO* displayed no constitutive expression. As a control, the blot was also stripped and rehybridized with a poplar actin probe. The actin probe was obtained from J. Patton. Actin mRNA

Figure 8. Northern analysis of *PtDFR* induction by mechanical wounding. Both wounded leaves and unwounded leaves on wounded plants (systemic) were harvested at the appropriate times, total RNA was extracted, and analyzed on RNA blots. Following hybridization with a $^{32}$P-labelled *PtDFR* probe, the blot was stripped and hybridized with poplar polyphenol oxidase (*PPO*) and actin. The *DFR* blot was exposed to X-ray film for 2 h, while both the *PPO* and actin blots were exposed for 24 h.


Wounded
Systemic
0 6 12 24 36 48 72
6 12 24 36 48 72 h
DFR
PPO
Actin
rRNA

expression was not induced by wounding, and appeared to remain constant for all samples. Thus, northern analysis indicates that simulated herbivory by mechanical wounding induces *PtDFR* mRNA expression both locally and systemically, and *PtDFR* expression was similar to the expression of other aspen defense genes.

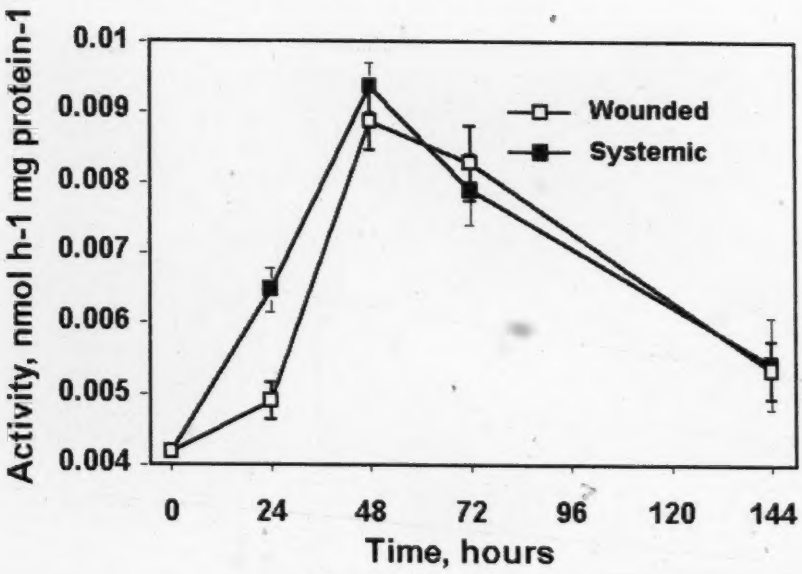
### *3.5 Induction of DFR Enzyme Activity by Wounding*

The wound-induced expression of *PtDFR* mRNA predicts that DFR activity should also increase following leaf damage. To detect DFR activity, the assay described by Stafford and Lester (1982) was adapted for trembling aspen. Initial attempts of performing the DFR assay on aspen leaf tissue were unsuccessful and a series of alterations and optimizations were performed. To improve the detection of DFR enzyme activity, frozen leaf tissue was not pre-extracted in acetone as stated by Stafford and Lester (1982). To test for linearity of reaction, enzyme extracts were incubated in the reaction mixture for 0.5 h to 6 h at 30 °C. Substrate was linearly converted to product over the first 1 h, and the rate of conversion decreased after this time. The greatest DFR activity, as measured in nmol substrate reduced $h^{-1}$ mg $protein^{-1}$, was detected after a 0.5 h incubation, and 0.5 h incubation times were used for all subsequent DFR activity assays. Controls included using boiled enzyme extracts for each DFR assay, which did not show DFR activity and controlled for any residual flavan-3,4-diols in the reaction tube. The assay was found to be repeatable as multiple DFR enzyme assays conducted with the same enzyme extracts resulted in almost identical DFR activity. Also, different enzyme extracts using the same leaf tissue resulted in similar DFR activity results for this assay. DFR activity was highest in enzyme extracts that had been freshly extracted, as

activity decreased by 10-15% in extracts stored at 4 °C for 1 day, and a 45-50% decrease in enzyme activity was observed for extracts that had been stored at 4 °C for 3 days.

DFR assays were performed on enzyme extracts from control, wounded and systemically wounded leaf samples in time course experiments. These experiments involved wounding the leaves of three aspen plants and harvesting one wounded and one systemic leaf from each plant at various times after wounding for individual analysis. Control samples were taken before wounding the aspen plants, and involved harvesting approximately one-quarter of each wounded aspen leaf from each aspen tree, and pooling samples from the same tree for protein extraction. Protein was extracted from each harvested leaf and used in DFR activity assays. DFR activity was found to increase 2-fold after wounding, peaking at 48 h and then declining (Figure 9). The induction of DFR activity was observed in both wounded and systemic leaves of aspen. Statistical analysis using ANOVA was used to compare DFR enzyme activity in wounded and systemically wounded leaves to DFR enzyme activity in control leaves from three separate aspen plants. This analysis suggested that the observed induction is significant after 48 h ($p=0.0004$) and 72 h ($p=0.002$) in wounded leaves, and after 48 h ($p=0.0001$) and 72 h ($p=0.002$) in systemic leaves. The kinetics of induction are consistent with the *PtDFR* mRNA induction, which is earlier and peaks at 24 h after wounding (see Figure 8). These experiments show that, like *PtDFR* mRNA, DFR enzyme activity is locally and systemically induced by wounding in aspen leaves.

Figure 9. Analysis of DFR activity in wounded and systemically wounded aspen leaves. Leaf proteins were extracted, desalted, and assayed for DFR activity as described in Materials and Methods. Each point is the mean of 3 plants assayed; bars represent the standard error.


Activity, nmol h-1 mg protein-1
0.01
0.009
0.008
0.007
0.006
0.005
0.004
0
24
48
72
96
120
144
Time, hours
Wounded
Systemic

*3.6 Wound Induction of Condensed Tannins in Aspen*

The induction of both *PtDFR* mRNA and DFR enzyme activity by simulated herbivory suggests that the ultimate products of the DFR enzyme, condensed tannins, may also increase in damaged leaves of aspen relative to control leaves. Furthermore, condensed tannin concentrations have been shown to increase in mechanically wounded aspen leaves (Osier and Lindroth, 2001). Thus, the concentration of condensed tannins in wounded leaf tissue from micropropagated aspen grown in the Biotron was assayed using the *n*-butanol:HCl assay. The observation of wound-inducible condensed tannins in aspen would be consistent with the DFR molecular data, and confirm observations made by other groups. As stated in 3.4, concentrations of condensed tannins are variable in the aspen clones used. Thus, in order to minimize this variability when measuring the wound induction of condensed tannins, many optimization experiments were performed to determine the leaf choice and sampling methods that would be used in time course experiments.

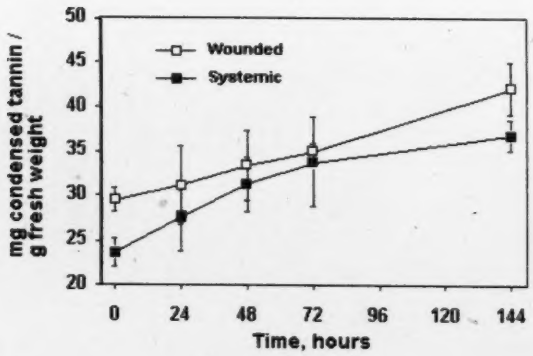
As stated in 3.4, experiments were performed to determine the variation in condensed tannins in aspen based on leaf position. Leaf series experiments suggested that the least variation in condensed tannin concentrations appears to be in leaves of LPI 9-21 as measured by the *n*-butanol:HCl assay. Thus, for wounding experiments, leaves of LPI 16-19 were chosen as the wounded leaves, and leaves of LPI 12-15 were designated as systemic samples. Furthermore, leaf-sampling methods were different for wounded and systemic samples. To determine the initial concentration of condensed tannins in wounded leaves, approximately one-quarter of the leaf was removed from the edge using a razor (used as a control sample), and the remaining edge of the leaf was

mechanically wounded using a hemostat. For systemic leaves, sampling experiments were performed to establish a method to determine the initial concentrations of condensed tannins in systemic samples without wounding the leaf, and inducing condensed tannin synthesis. Removal of a leaf disc with a size 8 corkborer, by taking care to avoid all primary and secondary leaf veins, was found to provide an accurate measure of condensed tannin concentration in systemic leaves. Experiments were performed to demonstrate that condensed tannins do not increase significantly after 72 h and 144 h in leaves of LPI 12-15 after corkborer treatment (ANOVA; df=3; $p>0.05$).

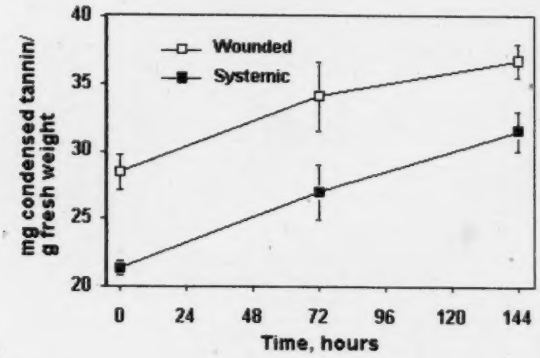
To determine if condensed tannins are induced by wounding in aspen leaves, eight trees of the same age and genotype were selected, and control samples for wounded (LPI 16-19) and systemic (LPI 12-15) leaves were taken before the wounding treatment as described above. Control samples of the same LPI from the eight trees were pooled for analysis. Leaves were wounded with a hemostat, and trees were kept in the Biotron growth chamber. Sampling of wounded leaves at each time point involved arbitrarily selecting five of the eight trees, and harvesting one leaf from each tree for individual analysis. The same procedure was repeated for sampling of systemic leaves. Analysis of leaf samples using the *n*-butanol:HCl assay revealed that condensed tannin concentrations increased over a 144 h period after wounding, in both the wounded and systemic leaves (Figure 10a). Some of the values had large standard errors due to the variation in constitutive concentrations of condensed tannins between different trees, though all were derived from the same clone. In the experiment shown, condensed tannin concentrations were significantly higher in wounded leaves both 72 h (ANOVA; df=4; $p=0.010$) and 144 h (ANOVA; df=4; $p=0.009$) after wounding compared to the control leaves. Condensed

Figure 10. Condensed tannin and anthocyanin concentrations in aspen leaves at different times after mechanical wounding. Acetone and methanol extracts were prepared and assayed as described in Materials and Methods. Each point is the mean of 5 plants assayed; bars represent the standard error. Condensed tannin concentrations are expressed in terms of relative equivalents to a sainfoin standard. 10a. *N*-butanol:HCl condensed tannin assays. 10b. Radial diffusion BSA precipitation assays. 10c. Anthocyanin concentrations from the same leaf samples as in 10a and 10b.

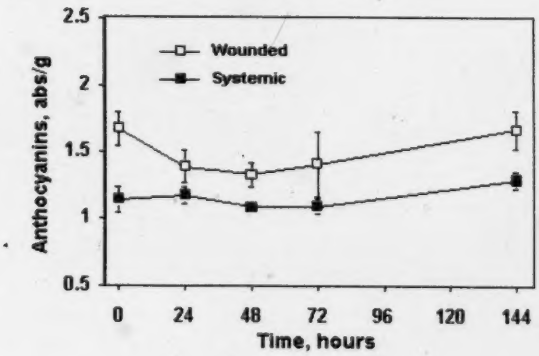
a


mg condensed tannin / g fresh weight
50
45
40
35
30
25
20
Wounded
Systemic
0
24
48
72
96
120
144
Time, hours

b


mg condensed tannin/ g fresh weight
40
35
30
25
20
Wounded
Systemic
0
24
48
72
96
120
144
Time, hours

c


Anthocyanins, abs/g
2.5
2
1.5
1
0.5
Wounded
Systemic
0
24
48
72
96
120
144
Time, hours

tannin concentrations were significantly higher in systemic leaves 144 h (ANOVA; df=4; p=0.001) after wounding compared to control leaves. A consistent increase in condensed tannin levels was observed after wounding in all experiments performed (Table 3), despite the observed tree-to-tree variation in absolute condensed tannin concentrations.

Condensed tannins are thought to function as an anti-nutritive defense by binding dietary protein and digestive enzymes in the herbivore gut. Therefore, a radial diffusion protein precipitation assay was also used as a second measure of condensed tannin concentrations (Hagerman, 1987). The ability of wounded leaf extracts to precipitate a test protein (bovine serum albumin) was assessed by this assay. Protein precipitation assays indicated that condensed tannin concentrations were higher in wounded and systemically wounded leaf extracts in comparison to control extracts (Figure 10b). In the experiment shown, condensed tannin concentrations were significantly higher in wounded (ANOVA; df=4; p=0.002) and systemic (ANOVA; df=4; p=0.0006) leaf samples 144 h after wounding. These results showed that the protein-precipitating capacity of acetone extracts from wounded and systemically wounded leaf samples is greater than control leaf extracts. In general, these radial diffusion assays gave results similar to the *n*-butanol:HCl assay. By observing condensed tannin induction using two completely different assay methods in many experiments, and showing that these increases in condensed tannin concentrations are consistent with increases in *DFR* mRNA accumulation and enzyme activity, the observed condensed tannin induction is considered to be real.

Table 3: Fold induction of condensed tannin concentrations in wounded aspen leaves 72 h and 144 h after wounding as measured after the butanol:HCl assay.

| Experiment | Fold Induction 72 h | 144 h |
|---|---|---|
| 1 | 1.89x | -- |
| 2 | 1.40x | -- |
| 3 | 1.62x | -- |
| 4 | 1.40x | 1.52x |
| 5 | 1.14x | 1.61x |
| 6 | 1.56x | 1.82x |
| 7 | 1.31x | 1.50x |

Since DFR is important in anthocyanin synthesis as well as condensed tannin synthesis, anthocyanin assays were performed on wounded and systemic leaf tissue to determine if tissue damage increased anthocyanin synthesis. Anthocyanin concentrations did not increase significantly in wounded and systemically wounded aspen leaf samples over the same time period that condensed tannin concentrations increased (ANOVA; df=4; $p>0.05$) (Figure 10c). In general, concentrations of anthocyanins initially declined, and then returned to constitutive levels or remained low. Anthocyanin induction was not observed. In conclusion, the wound response in aspen appears to lead to flavonoid precursors being predominantly channeled down the branch of the phenylpropanoid pathway leading to condensed tannin synthesis rather than anthocyanin synthesis.

*3.7 Wound-Induced Expression of the Phenylpropanoid Pathway*

Since previous results indicate that an increase in condensed tannin synthesis occurs following wounding, upregulation of the whole phenylpropanoid pathway is expected. To test this hypothesis, the expression of several key phenylpropanoid genes was investigated in response to wounding. The expression of phenylalanine ammonia-lyase (*PAL*), 4-coumarate-CoA ligase (*4CL*) and chalcone synthase (*CHS*) have previously been shown to be induced by wounding and stress treatments in other plant species (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995). These genes encode enzymes that are involved in metabolizing phenylalanine and malonyl-CoA to flavonoid molecules (Figure 1). Expression of the *PAL*, *4CL* and *CHS* genes is expected to be induced by wounding to increase the synthesis of enzymes that provide the flavonoid monomers required for condensed tannin synthesis. Recently, a cDNA was cloned from

*Arabidopsis* that is predicted to encode leucoanthocyanidin reductase (LAR), the first enzyme committed to condensed tannin synthesis (Devic *et al.*, 1999). Cloning this gene from aspen and using *LAR* as a probe in northern analysis is important in showing that genes specifically involved in condensed tannin synthesis are induced by wounding. Furthermore, the enzyme anthocyanidin synthase (ANS) is specific to anthocyanin synthesis and is involved in synthesizing anthocyanidins from flavan-3,4-diols (Pelletier *et al.*, 1997). The expression of *ANS* was studied to determine if enzymes involved in anthocyanin synthesis were downregulated after wounding as the enzymes involved in condensed tannin synthesis were upregulated, since anthocyanin concentrations were shown not to be induced in wounded aspen leaves (see 3.7).

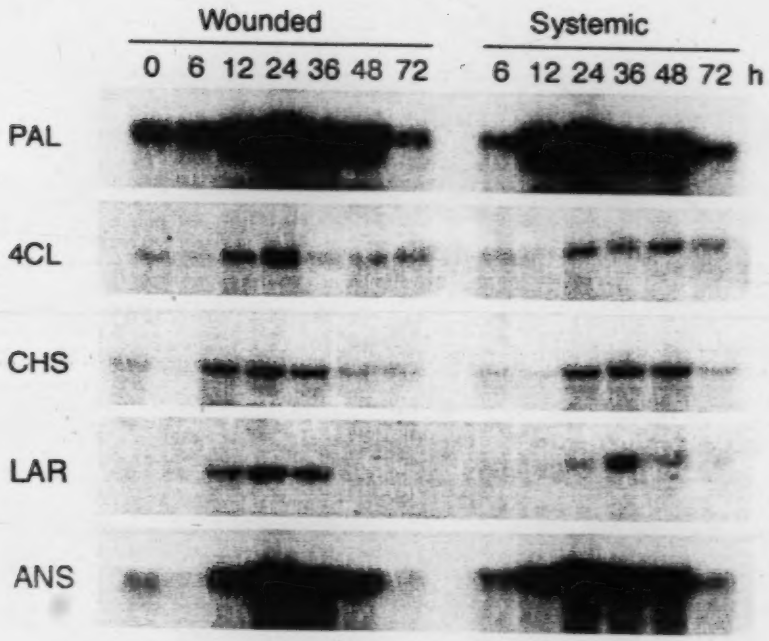
Probes for *PAL*, *4CL*, *CHS*, *LAR* and *ANS* were cloned from an aspen cDNA library by PCR using sequence information in Genbank and PopulusDB (http://poppel.fysbot.umu.se/), a searchable database of hybrid poplar sequences. Sequences of PCR primers used to clone each of the phenylpropanoid genes are presented in Table 1, and annealing temperatures for all primers were optimized using a gradient PCR thermocycler. *CHS*, *ANS* and *LAR* were previously not cloned in aspen, and novel sequence data for these genes is included in Appendix 3. The identities of the probes were confirmed by sequencing and comparison to sequences in Genbank using the BLAST search tool. The *PAL* sequence from aspen had the highest identity with phenylalanine ammonia-lyase from *Populus trichocarpa* x *P. deltoides* (96% identity) and *4CL* had the highest identity with 4-coumarate CoA:ligase from trembling aspen (100% identity). *CHS* from aspen had the highest identity with chalcone synthase from

*Betula pendula* (92% identity), and aspen *ANS* was most similar to leucoanthocyanidin dioxygenase from *Vitis vinifera* (81% identity).

Of particular interest was the cloning of *LAR*, which is a *DFR*-like gene that is specifically involved in producing flavan-3-ols for condensed tannin synthesis. *LAR* was cloned from aspen by designing primers based on sequences from the PopulusDB that have the highest identity with the *BAN* gene from *Arabidopsis*. Products that were produced from PCR with the LAR primers and aspen cDNA library were cloned into T-Vector and sequenced. The *LAR* sequence from aspen was compared to sequences in Genbank using the BLAST search tool and found to have the highest similarity with *BAN* from *Arabidopsis* (70% identity), followed by high similarities with *DFR* genes from a number of plant species (52%-54% identities). Sequence comparison with *PtDFR* revealed that *LAR* from aspen has a 58% sequence identity with *PtDFR*. Thus, *LAR* from aspen is not expected to cross-hybridize with *PtDFR* when the Southern blots are washed in high stringency washes, but may represent some of the *DFR*-like bands that appear when Southern blots are washed in low stringency washes.

Each of the phenylpropanoid partial sequences was used as probe in northern analysis with previous time course experiments. All five genes were wound-induced with kinetics similar to *DFR* (Figure 11). The kinetics of induction for all of the phenylpropanoid genes investigated were also similar, with maximum mRNA expression at 24 h after wounding, followed by a decline in expression to constitutive levels after 72 h. In systemic tissue, mRNA levels of *PAL*, *4CL* and *ANS* reached a maximum at 24 h, while expression levels of *CHS* and *LAR* were highest after 36 h. Additionally, *PAL*, *4CL*, and *CHS* had higher levels of constitutive expression at 0 h compared to *PtDFR*

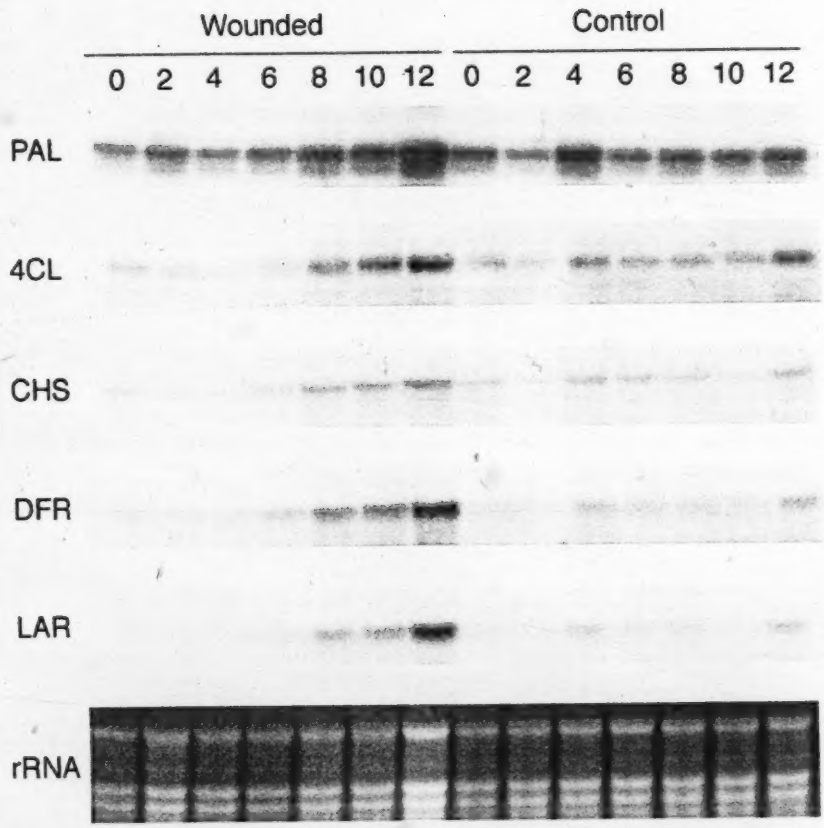
Figure 11. Northern analysis of the expression of key phenylpropanoid enzymes following mechanical wounding of aspen. Leaves were treated and analyzed as in Figure 8, and blots were stripped and reprobed with each phenylpropanoid cDNA fragment. Blots were probed with phenylalanine ammonia-lyase (*PAL*), 4-coumarate:CoA ligase (*4CL*), chalcone synthase (*CHS*), leucoanthocyanidin reductase (*LAR*), and anthocyanidin synthase (*ANS*). The *PAL* blot was exposed for 18 h, and the *4CL*, *CHS*, *LAR*, and *ANS* blots were exposed for 24 h. The *PAL* and *ANS* blots were exposed on X-ray film, while the *4CL*, *CHS* and *LAR* blots were exposed on a phosphorimager screen.


Wounded
Systemic
0 6 12 24 36 48 72
6 12 24 36 48 72 h
PAL
4CL
CHS
LAR
ANS

and *LAR*, likely a result of their role in the synthesis of other phenylpropanoids such as lignins or phenolic glycosides. Furthermore, a decrease in *4CL* and *CHS* mRNA was repeatedly observed after 4-6 h in wounded tissue. This decrease in *4CL* and *CHS* mRNA expression was observed in three separate experiments, and appears to be genuine in Figure 11, as these blots were reprobed from Figure 8, which shows even loading of RNA in each lane and even expression of the control probe actin at 0 h and 6 h. Surprisingly, expression of *ANS* mRNA was also induced by wounding with kinetics similar to *PtDFR* induction. Anthocyanin concentrations were not shown to be induced following wounding (Figure 10c), thus *ANS* mRNA upregulation does not seem to result in the accumulation of the end product of anthocyanin synthesis. Overall, the induction pattern observed in Figure 11 clearly suggests a coordinate upregulation of enzymes involved in phenylpropanoid metabolism.

To determine if genes involved "early" in the phenylpropanoid pathway are coordinately induced before genes involved "late" in the pathway, a short-term time course experiment was conducted to elucidate the kinetics of induction of phenylpropanoid genes between 0 h and 12 h after wounding (Figure 12). Previous studies have found that enzymes functioning near the beginning of the phenylpropanoid pathway are coordinately induced by light before the induction of genes involved "late" in the pathway, which exhibit distinct patterns of expression (Kubasek *et al.*, 1998). Samples were collected from unwounded plants to control for temporal changes in phenylpropanoid gene expression as a result of circadian rhythms or other conditions (Figure 12). Wounded and control samples were harvested every 2 h for 12 h and total RNA was isolated and analyzed by northern hybridization with *PAL*, *4CL*, *CHS*, *PtDFR*

Figure 12. Northern analysis of the expression of phenylpropanoid enzymes in mechanically wounded and unwounded (control) plants. Leaves were harvested at the appropriate times and analyzed as described in Materials and Methods. Blots were stripped and reprobed with each phenylpropanoid cDNA fragment; phenylalanine ammonia-lyase (*PAL*), 4-coumarate:CoA ligase (*4CL*), chalcone synthase (*CHS*), dihydroflavonol reductase (*DFR*), leucoanthocyanidin reductase (*LAR*), and anthocyanidin synthase (*ANS*). All blots were exposed for 24 h on a phosphorimager screen.


Wounded
Control
0 2 4 6 8 10 12 0 2 4 6 8 10 12
PAL
4CL
CHS
DFR
LAR
rRNA

and *LAR*. All five phenylpropanoid genes displayed induced expression following wounding after 12 h. *PAL*, *4CL* and *CHS* all displayed high constitutive expression compared to *PtDFR* and *LAR*. *PAL* appeared to be induced at 6 h after wounding. Expression of both *4CL* and *CHS* initially declined 4 h after wounding, and then increased up to 12 h. The decrease in *4CL* and *CHS* expression at 4 h appears to be shifted back in time in Figure 12 when compared to the 6 h decrease in expression observed for *4CL* and *CHS* in Figure 11. *DFR* and *LAR* displayed similar patterns of expression with induction occurring 6 h after wounding. Overall, genes involved "early" in the phenylpropanoid pathway did not show conclusive induction before genes involved "late" in the pathway after wounding.

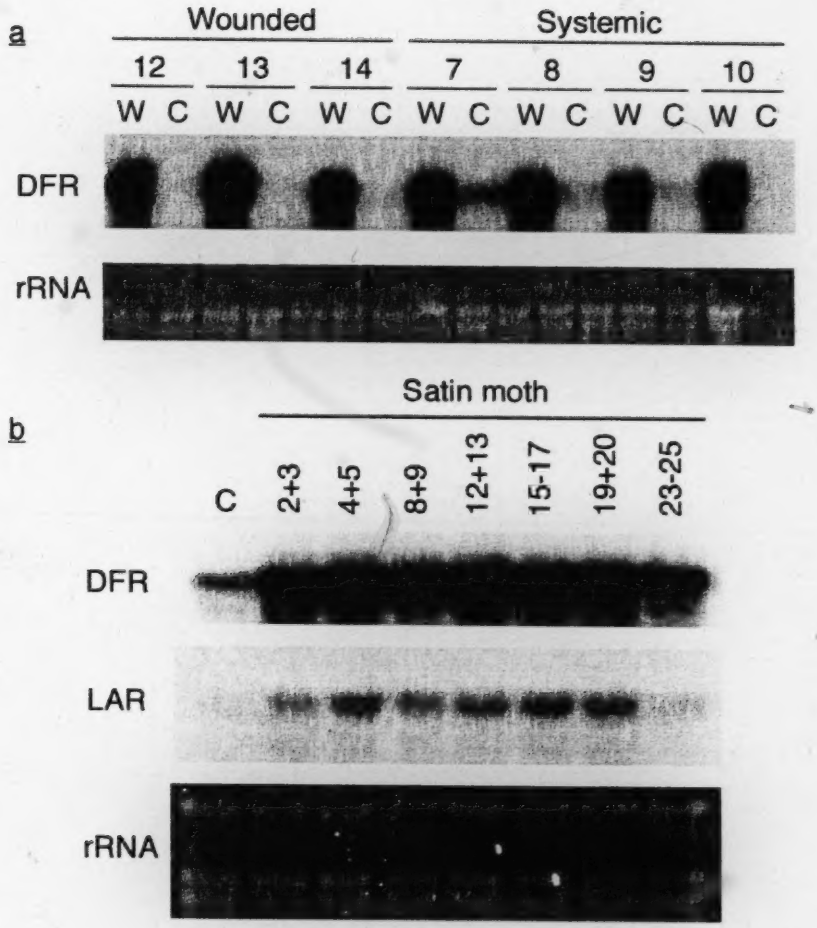
For the control experiment, all genes appeared to have the same pattern of expression. The 0 h sample was taken 2 h after the lights in the growth chamber had been turned on, and the 12 h sample was taken 3 h before the lights in the growth chamber were turned off. All genes had the same constitutive expression as observed in the wounding experiment, though there appeared to be a slight decrease in expression in all genes at 2 h, which appeared to increase to slightly higher levels at 4 h compared to constitutive levels (0 h). Expression then declined slightly and appeared to plateau before increasing once again at 12 h. The increased expression of *4CL* and *CHS* at 4 h in the control experiment contrasts with the apparent decrease in expression observed in the wounding experiment. In conclusion, all phenylpropanoid genes had the same general pattern of expression in the control blot of Figure 12, which included slight variations in

expression over the 12 h that phenylpropanoid gene expression was observed. However, these patterns of expression do not appear to fit a circadian rhythm (see Discussion).

*3.8 Herbivore and Methyl Jasmonate Induction of DFR Expression*

Mechanical wounding that was designed to simulate chewing insect damage was shown to induce *PtDFR* mRNA expression, thus *PtDFR* appears to be important in defense in aspen. In order to demonstrate that *PtDFR* expression is induced by insect herbivore damage, two different species of insect larvae that feed on the leaves of *Populus* species were used in herbivore experiments. One of the insect herbivores used was the forest tent caterpillar (*Malacosoma disstria* Hbn.), which is a major defoliator of aspen forests and whose populations' outbreak with a periodicity of about ten years (Duncan and Hodson, 1958). Also larvae of the satin moth (*Leucoma salicis* L.), an introduced pest that generally feeds on *Populus* species, were used in experiments. Leaves of LPI 12-17 of two three-month old aspen plants were subjected to damage by forest tent caterpillars (FTC) in an insect cage (see Materials and Methods), which resulted in removal of approximately 10-25% of the leaf area. Damaged leaves, as well as undamaged (systemic) leaves of LPI 7-10, were harvested 24 h after wounding and analyzed by northern hybridization with the full-length *PtDFR* probe. *PtDFR* mRNA was present at low levels in leaves of two separate control plants that were also placed within an insect cage in the growth chamber (Figure 13a, C). On the other hand, *PtDFR* mRNA accumulated to high concentrations in both wounded and unwounded leaves on aspen trees damaged by forest tent caterpillar larvae (Figure 13a, W). The degree of *PtDFR* induction by FTC was comparable to that observed for mechanical wounding.

Figure 13. Northern analysis of *DFR* and *LAR* expression in leaves following herbivore damage. Total RNA was extracted and analyzed by RNA blots as described in Materials and Methods. 13a. Forest tent caterpillar (FTC) larvae fed on leaves of LPI 12-14, and the damaged and undamaged leaves (leaves of LPI 7-10; systemic) on wounded (W) trees were harvested 24 h after feeding. Identical leaves (LPI 7-14) from an unwounded plant were used as a control (C). 13b. Satin moth larvae fed continuously on aspen plants for 96 h, and leaves (LPI 2-25) were grouped by age categories for analysis. RNA was also extracted and analyzed from pooled control leaves (C) from each age category.


a
Wounded
Systemic
12
13
14
7
8
9
10
W C W C W C W C W C W C W C
DFR
rRNA
b
Satin moth
C
2+3
4+5
8+9
12+13
15-17
19+20
23-25
DFR
LAR
rRNA

Herbivore experiments with satin moth (*Leucoma salicis* L.) larvae were also conducted in insect cages. In this experiment, the larvae were allowed to freely move and feed on an entire aspen plant for 96 h. The caterpillar larvae showed a preference for older leaf tissue (leaves 8-12), as many of the young leaves remained uneaten (leaves 2-5). Leaves of several age classes were harvested for northern blot analysis, and *PtDFR* mRNA expression was found to be induced to high concentrations in all leaves on wounded plants compared to control leaves, which were obtained from an aspen plant of the same age that had also been placed in an insect cage for 96 h (Figure 13b). Continuous feeding by satin moth larvae appeared to induce *PtDFR* expression to higher levels than those seen in the mechanical wounding (Figure 7) and FTC experiments (Figure 13a). Furthermore, the blot was stripped and probed with *LAR* (Figure 13b), which also appeared to be induced to higher levels than those seen in mechanical wounding experiments (Figure 11). Thus, *PtDFR* mRNA expression is induced locally and systemically by both aspen herbivores, indicating that *PtDFR* expression is part of the induced defense response against insects. Furthermore, *LAR* expression was also induced following insect damage, which suggests that condensed tannin synthesis is an important defense response to insect herbivory.

Methyl jasmonate is a plant hormone that is widespread in the plant kingdom and regulates many physiological processes, including plant defense responses (Farmer and Ryan, 1990; Weiler, 1997). Previously, methyl jasmonate treatments were found to induce the expression of trypsin inhibitor and polyphenol oxidase genes in trembling

aspen (Haruta et al., 2001a; Haruta et al., 2001b). Since *PtDFR* expression is induced by both wounding and insect herbivory, methyl jasmonate treatment is also expected to induce *PtDFR* expression, as jasmonates are signaling compounds that operate in the defense response. The effect of methyl jasmonate treatment on *PtDFR* expression in aspen leaves was investigated by probing RNA from leaves of young (4 week-old) and old (12 week-old) aspen plants treated with methyl jasmonate. *PtDFR* expression was induced in both the young and old methyl jasmonate-treated leaves (Figure 14). Also, young leaves showed a stronger induction of *PtDFR* compared to old leaves. Thus, wound-induced *PtDFR* mRNA expression may be regulated by jasmonate signaling in aspen, similar to other known aspen defense genes.

In conclusion, *PtDFR* expression is induced following mechanical wounding, herbivore damage and methyl jasmonate treatment. This evidence indicates that *PtDFR* expression is part of the defense response in aspen.

### *3.9 Expression of DFR in Aspen Tissues*

Condensed tannins are found constitutively in both leaf and woody tissues of trembling aspen (Lindroth and Hwang, 1996). In order to compare *PtDFR* gene expression with the appearance of condensed tannins in aspen tissues, the constitutive expression of *PtDFR* was measured in several aspen tissues by northern hybridization. Constitutive *PtDFR* mRNA expression was found in untreated leaves, petioles, stems and roots of aspen plants growing in growth chambers (Figure 15). Condensed tannins were also detected by the *n*-butanol:HCl method in these tissues, with the exception of

Figure 14. Northern analysis of *PtDFR* expression in leaves following methyl jasmonate treatment. Both young and old aspen plants were treated with methyl jasmonate (MeJ) for 24 h, and leaves were harvested. Untreated leaves from young and old leaves were also harvested as a control (C).

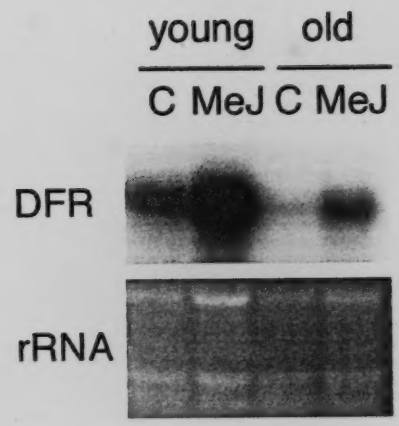
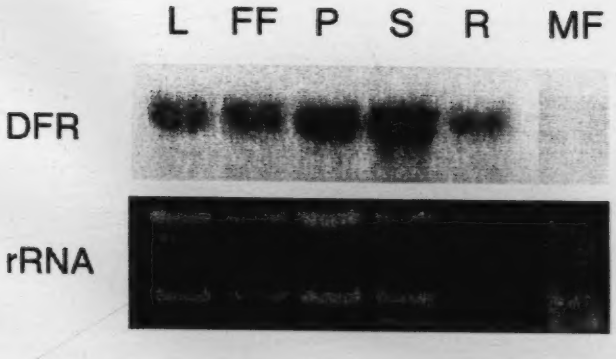

young
old
C MeJ C MeJ
DFR
rRNA

Figure 15. Developmental expression of *DFR* in unwounded plants. L, leaves; P, petioles; S, stems; R, roots. FF and MF refers to male and female flowers from outdoor grown trees, respectively.


L FF P S R MF
DFR
rRNA

petioles, which contained high concentrations of anthocyanins (Table 4). Additionally, *PtDFR* mRNA expression was demonstrated in female, but not male flowers of outdoor grown aspen trees. Thus, *PtDFR* appears to be constitutively expressed at low concentrations throughout saplings of trembling aspen, and constitutive *PtDFR* expression levels are associated with the appearance of condensed tannins in aspen tissues.

*3.10 Comparative DFR Expression in Populus trichocarpa x P. deltoides*

Hybrid poplar has previously been shown to exhibit a strong inducible defense response that includes the expression of *PPO* and trypsin inhibitor genes (Constabel *et al.*, 2000). As a comparison, the induction of *PtDFR* in mechanically wounded and FTC-damaged leaves of trembling aspen was compared to that in hybrid poplar. This experiment would give evidence as to whether *PtDFR* expression and condensed tannin synthesis is important as a general defense response in *Populus* species, or whether *PtDFR* mRNA induction is specific to aspen. Surprisingly, *PtDFR* induction in hybrid poplar was very low compared to trembling aspen (Figure 16, top panel). *PtDFR* is expected to strongly hybridize with *DFR* from hybrid poplar, as hybrid poplar *DFR* sequences, obtained from hybrid poplar EST projects (M. Christopher and C.P. Constabel, unpublished data), were 90% identical to *PtDFR*. The blot was stripped and reprobed with *PPO* to confirm that hybrid poplar was responding strongly to both the mechanical and herbivore damage. Strong *PPO* induction was observed in both trembling aspen and hybrid poplar (Figure 16, bottom panel). Therefore, strong *PtDFR* induction may only be a characteristic of *Populus tremuloides*, and may not be found in

Table 4: Measurements of condensed tannin and anthocyanin content in various tissues from trembling aspen. Condensed tannins were quantified using the butanol:HCl method. Anthocyanins were extracted in acidic methanol, and the absorbance determined and corrected at 530 nm. Measurements were performed on at least three samples for each tissue and the mean values are shown.

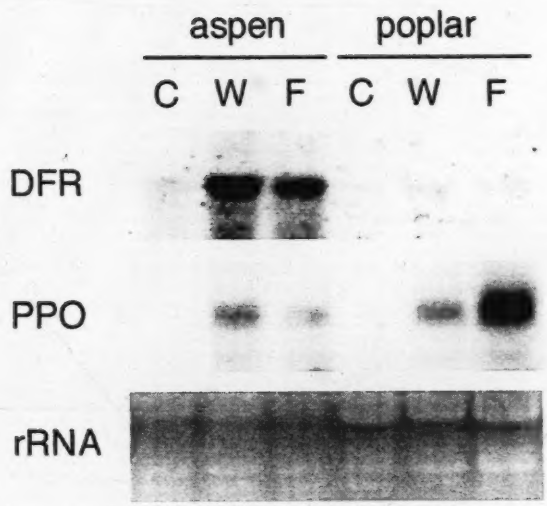
**Condensed Tannins**

| **Aspen Tissue** | **Concentration (mg $g^{-1}$ fresh weight)** |
|---|---|
| Petioles | 0 |
| Stems | 4.2 |
| Roots | 7.3 – 37.1[1] |
| Female flowers | 9.4 |
| Male flowers | 2.7 |

| **Anthocyanins** | **Abs $g^{-1}$ fresh weight** |
|---|---|
| Petioles | 2.8 |
| Stems | 1.6 |
| Roots | 0.2 |
| Female flowers | 4.4 |
| Male flowers | 13.0 |

[1]- A range of condensed tannin concentrations is listed for aspen roots as condensed tannin concentrations were found to be highest in root sections taken within 5 cm of the root tip (up to 37.1 mg $g^{-1}$ fresh weight), and lower for root sections taken 5-20 cm from the root tip.

Figure 16. Comparative analysis of *DFR* and *PPO* induction in aspen and poplar plants. Plants were induced for 24 h; C, control; W, mechanically wounded; F, FTC damaged. All blots were exposed for 24 h.


aspen
poplar
C W F C W F
DFR
PPO
rRNA

other *Populus* species. This is consistent with condensed tannin data, as hybrid poplar had low foliar concentrations of condensed tannins (1-2 mg condensed tannin/g fresh weight), which did not appear to be inducible by mechanical wounding. Thus, hybrid poplar may rely more on protein-based defense, while aspen has evolved inducible phytochemical defenses.

# 4. Discussion

The defensive role of condensed tannins has been widely studied in many plant species. Condensed tannins have been implicated as an inducible defense in a few plant species, though no studies have investigated the molecular events that occur during condensed tannin induction. In this study, trembling aspen (*Populus tremuloides*), a forest tree species that produces large quantities of condensed tannins in its leaves, was used to study wound-induced accumulation of condensed tannins and the expression of phenylpropanoid genes during this defense response. Specifically, a cDNA for the enzyme dihydroflavonol reductase (DFR) was cloned and sequenced from trembling aspen, as this is a key enzyme in condensed tannin synthesis. The expression of *PtDFR* was characterized and found to be induced by wounding, herbivore damage and methyl jasmonate treatment in aspen leaves. Furthermore, the activity of DFR was shown to increase following wounding, along with an increase in the concentrations of condensed tannins. Thus, *PtDFR* induction appears to be important in the defense response of aspen.

## *4.1 Analysis of DFR and DFR-like Genes in Aspen*

The cloning of *PtDFR* from trembling aspen is the first report of a full-length *DFR* being cloned and sequenced from a forest tree species. DFR is involved in the late stages of the synthesis of condensed tannin monomers, and genetic manipulation of the expression of *DFR* mRNA has been shown to directly affect condensed tannin synthesis in *Lotus corniculatus* (Carron *et al.*, 1994, Robbins *et al.*, 1998). Sequence analysis of

*PtDFR* revealed a conserved NADPH-binding domain, and high similarity to *DFR* sequences from other plant species. Furthermore, analysis by PSORT predicted that *PtDFR* is peripherally associated with membranes, which is consistent with DFR being part of an enzyme complex with chalcone synthase (CHS) and chalcone isomerase (CHI) that is localized at the endoplasmic reticulum and vacuolar membrane (Burbulis and Winkel-Shirley, 1999; Saslowsky and Winkel-Shirley, 2001). Examination of the proposed substrate specificity region of *PtDFR* reveals that asparagine-134, a conserved residue among most DFR proteins, has been substituted with an aspartic acid residue in PtDFR. This amino acid position in the DFR protein appears to be important in substrate specificity, as site-directed mutagenesis of asparagine-134 to leucine resulted in a *Gerbera* DFR enzyme with altered substrate preferences; a DFR enzyme that preferentially reduced dihydrokaempferol (4' hydroxylation on B-ring) (Johnson *et al.*, 2001). *Petunia* DFR, which like aspen DFR, also contains an asparagine residue at position 134, is unable to reduce dihydrokaempferol (Johnson *et al.*, 2001). This suggests that PtDFR may also inefficiently reduce dihydrokaempferol, and have a higher affinity for dihydroquercetin and dihydromyricetin (3',4'-hydroxylation, and 3',4',5'-hydroxylation patterns on the B-ring respectively). Preference for these highly hydroxylated dihydroflavonols would be consistent with a defensive role for DFR, as greater hydroxylation in condensed tannins allows for greater chemical bond formation and stronger protein precipitation, which is correlated with anti-herbivore potential (Ayres *et al.*, 1997). The substrate preference of PtDFR could be tested using the recombinant PtDFR enzyme expressed in *E. coli*, with the proper chemical substrates.

A second DFR from aspen (*PtDFR-2*) was cloned using a PCR-based approach. Sequence analysis of *PtDFR-2* predicted 18 amino acid differences between PtDFR and PtDFR-2 (Figure 4). Three of these amino acid differences were present in the substrate specificity region (K138H, R139K, D152E), though these amino acid differences appear to be conserved, as amino acids with similar polarity in their side chains appear at all three variant positions. Site-directed mutagenesis of these three amino acid positions to hydrophobic amino acids has been performed in *Gerbera* DFR, and found to result in DFR enzymes with unaltered substrate preferences (Johnson *et al.*, 2001). Thus, PtDFR and PtDFR-2 are predicted to have similar substrate preferences despite the amino acid differences between the two enzymes.

Many plant species contain small gene families in their genomes that encode structural genes of the phenylpropanoid pathway. DFR has been found to occur as both single genes and multi-gene families in various plant species (see 1.4.1 for references). Southern blots washed in high stringency conditions revealed only one hybridizing *PtDFR* band in aspen genomic DNA, which suggests that the aspen genome contains a single copy of *DFR* (Figure 6a). There were polymorphisms between the two aspen clones analyzed, though both clones only exhibited one single *DFR* band in Southern blots washed in high stringency conditions (data not shown). Both *PtDFR* and *PtDFR-2* are expected to remain hybridized to the *PtDFR* probe after high stringency washes, as these sequences are 89% identical. Since only one band appears on Southern blots, *PtDFR* and *PtDFR-2* are expected to be alleles of the single copy of *DFR* in the aspen genome. For comparison, hybrid poplar (*Populus trichocarpa* x *Populus deltoides*) genomic DNA was also analyzed by Southern blotting with the *PtDFR* probe, and two

*DFR* bands appeared on blots washed in high stringency conditions (Figure 7). These two bands likely represent the two distinct parental *DFR* alleles in the hybrid. Overall, these results suggest that a single *DFR* locus is present in *Populus*.

The presence of faint bands seen on *PtDFR*-probed membranes washed in low stringency conditions suggests that other *DFR*-like genes may be present in the aspen genome. Specifically, the *BAN* gene from *Arabidopsis* was identified as a *DFR*-like gene that has a conserved NADPH-binding region (Devic *et al.*, 1999), and 52%-54% identity to *DFR* sequences from other plant species. The *BAN* gene is predicted to encode the enzyme leucoanthocyanidin reductase (LAR), which acts downstream of DFR in condensed tannin synthesis by reducing leucoanthocyanidins to catechins. A *DFR*-like sequence with high similarity to *BAN* (70% identity) was cloned from aspen (*LAR*), and found to be 58% identical to *PtDFR*. The aspen *LAR* sequence is expected to appear hybridized with *PtDFR* on Southern blots washed in low stringency conditions, but not high stringency conditions. Thus, the faint bands that appear on Southern blots of aspen genomic DNA probed with *PtDFR* and washed at low stringency are expected to include *LAR*, which has a related function to aspen DFR, and possibly other similar sequences that have the conserved NADPH-binding region (Ostergaard *et al.*, 2001).

*4.2 Induction of Defense Genes in Aspen*

Northern analysis revealed that *PtDFR* expression is induced by wounding and herbivore damage in aspen leaves (Figure 8 and 13a,b). Previously, *DFR* expression was shown to be induced by wounding in detached corollas of *Petunia* (Tamari *et al.*, 1995). The wound-induced expression of *PtDFR* is similar to the expression of other defense

genes in aspen. *PtDFR* displayed the same induction pattern as *PPO* (Figure 8), which is a known herbivore-induced defense gene in both poplar and aspen (Constabel *et al.*, 2000; Haruta *et al.*, 2001b). Furthermore, *PtDFR* induction appears to be similar to the expression of trypsin inhibitor genes in aspen, which also showed peak expression 24 h after wounding (Haruta *et al.*, 2001a). Since *PtDFR* gene expression is similar to the wound-induced expression of other known aspen defense genes, *PtDFR* is likely part of the aspen defense response against herbivory.

*DFR* induction by wounding and herbivory suggests that condensed tannins, compounds that appear to be important in aspen defense (Traw *et al.*, 1996; Hemming and Lindroth, 1999), may be inducible. The accumulation of *PtDFR* mRNA after wounding is followed by increased DFR enzyme activity in wounded leaves, and higher concentrations of condensed tannins. *PtDFR* mRNA induction peaks at 24 hours after wounding, and DFR enzyme activity was observed to peak at 48 hours following wounding. The DFR enzyme is involved in providing the flavonoid precursors for condensed tannin synthesis, and the increased DFR enzyme activity appears to lead to increases in the concentrations of condensed tannins over the 144 h that condensed tannins were measured. Of interest, *PtDFR* mRNA levels increased 7-10 fold over constitutive levels, while both DFR enzyme activity and condensed tannin concentrations increased approximately two-fold. This difference may be due to the high constitutive levels of both DFR activity and condensed tannin concentrations, which may have diluted the effects of the wound induction.

*4.3 Wound-Induced Condensed Tannin Accumulation in Aspen*

Condensed tannins are proposed to be defensive compounds in some plant species, and stress-induced accumulation of condensed tannins has been observed in a few plants, including trembling aspen (Osier and Lindroth, 2001). In this research, the wound-induced accumulation of condensed tannins in aspen leaves was observed in many replicate experiments by two different methods of measuring tannins. In preliminary experiments, control samples were taken from all of the leaves on an aspen tree, and the leaves were subsequently wounded and harvested for condensed tannin analysis after 72 h. Assuming that constitutive concentrations of condensed tannins remain constant with time, condensed tannin concentrations were higher 72 h after wounding in the same leaf as the control sample was taken in all experiments. This led to the design of time course experiments involving multiple plants and additional time points. In all experiments, condensed tannin concentrations were higher 72 h and 144 h after wounding (Table 3). However, values for condensed tannin concentrations displayed large standard errors at each time point due to the variability in constitutive concentrations of condensed tannins between trees. Growth conditions and sampling methods were optimized to reduce the variability in condensed tannin levels. Aspen trees were grown in soil containing slow-release fertilizer in fixed amounts, as nutrients, such as nitrogen, have been shown to have an effect on condensed tannin concentrations. Specifically, condensed tannin concentrations have been shown to increase in aspen leaves in response to low soil nitrogen (Kinney *et al.*, 1997). According to the carbon-nutrient balance hypothesis, nutrient limitation is hypothesized to decrease growth more than photosynthesis resulting in the accumulation of excess carbohydrates, which are

subsequently diverted to storage and defense compounds, thus increasing tissue carbon:nitrogen ratios (Bryant *et al.*, 1983). In addition to nutrients, care was taken to ensure that trees were given adequate space in the growth chamber so that individual aspen plants received the same access to light, which has also been shown to affect condensed tannin concentrations. Even though growth conditions were carefully monitored for aspen plants, individual trees of the same clone still displayed variation in condensed tannin concentration.

In order to determine if the induced concentrations of condensed tannins in wounded leaves were statistically greater than the condensed tannin concentrations in control leaves, ANOVA was performed. Single factor analysis of variance was performed to determine if the mean condensed tannin concentration in wounded leaves was significantly different than the mean condensed tannin concentration in control leaves. Since one leaf was removed from each sampled tree at each time point, ANOVA analysis could be performed with four degrees of freedom, as five trees were selected at each time point (df =n-1). In the experiment shown (Figure 10), the mean condensed tannin concentration in wounded and systemic leaves after 144 h was significantly different than the concentration of condensed tannin in control samples. Though the effects of wounding was not significant in all experiments, condensed tannin concentrations tended to be higher in wounded leaves harvested at 144 h, which is consistent with the wound-induced increases in *DFR* mRNA and DFR enzyme activity. The statistical analysis of induced condensed tannins could have been improved by adopting a better sampling strategy. Wounded leaves from each aspen tree should have been designated for sampling at a specific time point so that after control samples have

been taken at 0 h, the wounded and systemic samples could be harvested at one time point (such as 144 h) for comparison. Thus, each tree would be one unit of replication, and not the harvested leaves, as in the method of sampling that was performed. Furthermore, controls for time must be incorporated into the experimental design by designating unwounded aspen plants for sampling at the same times that leaves from wounded plants are harvested.

To strengthen the observation that condensed tannins are induced by wounding in aspen leaves, two distinct assay methods were used to measure condensed tannins. The *n*-butanol:HCl method is specific for condensed tannins and is generally regarded as the best assay for the determination of condensed tannin concentrations (Hagerman and Butler, 1989). Similar condensed tannin accumulation was also observed using the BSA radial diffusion method, which measures the formation of insoluble tannin-protein complexes *in vitro* and is particularly suitable for the analysis of large numbers of samples (Hagerman, 1987). Since condensed tannins are considered defensive in nature due to their ability to bind or precipitate protein, evidence in this research suggests that wound-induced aspen leaves will be a lower quality food source for herbivores than control leaves. The radial diffusion assay could be modified to use proteins such as those found in the diets of herbivores to unequivocally show that wound-induced aspen leaves have greater protein-precipitating, defensive capacities than control leaves.

Values for condensed tannins were expressed in terms of the sainfoin standard. Sainfoin condensed tannin was used as a standard because of the difficulty in finding a simple extraction procedure that would yield chemically pure aspen tannin. A method for purifying condensed tannins was obtained and found to be very laborious; too lengthy for

the scope of this project (Hagerman and Butler, 1980). Thus, purified condensed tannin from sainfoin was obtained as a gift, and relative amounts of condensed tannin were determined and reported in terms of the weight of the sainfoin standard. Measurements of tannins using other assays, such as the Folin-Denis assay, are significantly affected by the standard used (Appel *et al.*, 2001). However, the *n*-butanol:HCl method is structurally specific for condensed tannins and is not expected to be significantly affected by the standard used.

Overall, two assays supported the observation that condensed tannins are induced by wounding in aspen. This is the first report that links the accumulation of condensed tannins to increases in DFR expression and activity in any plant species.

### *4.4 Upregulation of the Phenylpropanoid Pathway by Wounding*

The phenylpropanoid pathway is responsible for synthesizing many compounds that protect the plant from abiotic and biotic stress (Dixon and Paiva, 1995). Plants constitutively produce certain phenylpropanoid compounds, such as lignin and flavonols, which supply structural support and protection from UV radiation. Thus, genes involved in synthesizing these constitutive phenylpropanoids are expected to be expressed constitutively at low levels in the plant. This hypothesis was confirmed as genes involved early in the general phenylpropanoid pathway were shown to exhibit higher levels of constitutive expression than those genes involved late in the pathway that produce more specific compounds (Figure 11). *PAL*, which encodes the first enzyme of the phenylpropanoid pathway and is responsible for providing the precursors for all other phenylpropanoids, had the highest background levels of expression, followed by *4CL* and

*CHS* expression, which demonstrated increased levels of constitutive expression compared to *PtDFR* and *LAR*. *PtDFR* may have shown low constitutive expression in most aspen tissues because DFR is only involved in the constitutive synthesis of condensed tannins and anthocyanins. All of the phenylpropanoid genes investigated demonstrated induced expression after wounding, thus the phenylpropanoid pathway appears to be involved in plant defense. As a comparison, many other plant defense genes, such as *PPO* and *TI*, do not exhibit constitutive expression, and *PPO* and *TI* mRNA is only detectable after wounding and herbivore attack (Constabel *et al.*, 2000; Haruta *et al.*, 2001a).

Wounding upregulated the expression of the general phenylpropanoid pathway as *PAL*, *4CL*, and *CHS* were induced in parallel to *PtDFR*, which confirms that phenylpropanoid biosynthesis is stimulated. The expression of *ANS* and *LAR* were also found to increase following wounding with similar kinetics to *PtDFR*. Interestingly, both *4CL* and *CHS* displayed decreased expression 4-6 h following wounding (Figure 11 and 12). This decreased expression may be due to the presence of multiple copies of *4CL* and *CHS* in the genomes of *Populus* species. Two differentially regulated, distinct genes for 4-coumarate:CoA ligase (*4CL*) are present in hybrid poplar (Allina *et al.*, 1998), and in trembling aspen, two *4CL* genes were isolated and the proteins found to share only 63% identity (Hu *et al.*, 1998). These 4CL proteins displayed divergent substrate specificity, and the *4CL1* isoform was expressed in lignifying tissues, while the *4CL2* isoform was expressed in stem and leaf tissues and expected to be involved in non-lignin phenylpropanoid synthesis. Furthermore, a total of four *CHS* genes were isolated from hybrid poplar (Lurin and Jouanin, 1995). Plants may be able to regulate phenylpropanoid

synthesis by possessing multiple copies of phenylpropanoid genes that are differentially expressed. Thus, during wound induction, it is possible that the observed decrease in *4CL* and *CHS* expression may be due to the downregulation of constitutive forms of these genes, followed by the upregulation of inducible forms that function in defense. This would allow the plant to control for the synthesis of specific phenylpropanoid precursors, as multiple isoforms of phenylpropanoid enzymes commonly have variable substrate specificities, and the wound-induction of a specific enzyme isoform would lead to the production of specific phenylpropanoids involved in defense.

The branch of the phenylpropanoid pathway leading to anthocyanin synthesis was expected to be downregulated by the wound response, so that *PtDFR* expression would lead solely to condensed tannin synthesis. However, *ANS* expression was shown to be induced by wounding, though anthocyanin assays revealed that anthocyanin concentrations did not increase significantly in wounded tissue. This observation could be explained by the presence of general regulatory factors that control the expression of genes involved "late" in the phenylpropanoid pathway. Although specific regulators of *DFR*, such as the *TT8* gene, have been reported (Nesi *et al.*, 2000), general regulators of "late" gene expression, such as the *TTG* regulatory gene are known to control the expression of both *DFR* and *ANS* (Pelletier *et al.*, 1997). Thus, wound-induction in aspen leaves may lead to the activation of regulatory factors that generally activate "late" genes in the phenylpropanoid pathway. It is possible that flavonoid precursors are compartmentalized in the cell and sequestered specifically for condensed tannin synthesis (Stafford, 1990). This would ensure that condensed tannin synthesis occurs following wounding and not the synthesis of other flavonoid compounds (such as anthocyanins).

The kinetics of induction of the phenylpropanoid genes were investigated between 0 h and 12 h following wounding, and in general, genes involved "early" in the pathway were not conclusively induced before genes involved "late" in the pathway. In order to determine whether "early" phenylpropanoid genes are induced prior to "late" genes, northern analysis of each of the phenylpropanoid genes could be conducted at 0.5 h intervals between 4-10 h after wounding. An oligonucleotide microarray analysis in *Arabidopsis* revealed that genes involved in the phenylpropanoid pathway had expression patterns that followed a circadian rhythm. The phenylpropanoid pathway was upregulated before dawn so that photoprotective compounds were available with the oncoming exposure to light (Harmer *et al.*, 2000). Based on these findings, phenylpropanoid gene expression was expected to be higher at 0 h (just after the lights had been turned on) and decline to 6 h before increasing once again to 12 h. In unwounded aspen leaves, all phenylpropanoid genes had the same general pattern of expression, which did not fit the circadian rhythm pattern of expression observed in *Arabidopsis* (Figure 12). This indicates that phenylpropanoid gene expression may be mediated by an unidentified source of variance that changes with time, and this source of variance needs to be reduced in future experiments. Phenylpropanoid gene expression in unwounded plants did not appear to display a specific pattern of expression, and more experiments at different times of the light-dark cycle would have to be performed in order to determine if phenylpropanoid gene expression follows a circadian rhythm pattern in aspen.

*4.5 Systemic Induction of PtDFR and Condensed Tannin Accumulation*

This research suggests that DFR is important in aspen defense, as *PtDFR* was shown to be coordinately induced along with other defense proteins, such as polyphenol oxidase (PPO; Figure 8) and trypsin inhibitor (TI; Haruta *et al.*, 2001a). Furthermore, this research has demonstrated that herbivore- and wound-induced *PtDFR* expression and condensed tannin accumulation appears to be a systemic response, like *PPO* and *TI* expression. Plants are able to convey localized damage to other undamaged parts by signal transduction, which results in the increased production of defensive compounds in undamaged tissues and systemic resistance. An important chemical signal is jasmonic acid, which accumulates in wounded tissues and has been shown to induce the expression of defense genes (Creelman *et al.*, 1992). Methyl jasmonate, a volatile ester of jasmonic acid, was shown to induce the expression of *PtDFR* in aspen, indicating that the signal transduction pathway leading to *PtDFR* expression includes endogenous jasmonate signals (Weiler, 1997). Methyl jasmonate treatment was also shown to induce the expression of *DFR* in detached corollas of *Petunia*, which led to the accumulation of anthocyanins (Tamari *et al.*, 1995). The induction of *PtDFR* by methyl jasmonate is coordinated with the induction of other defense genes in aspen, as both *PPO* and *TI* expression are also induced by methyl jasmonate treatment (Haruta *et al.*, 2001a; Haruta *et al.*, 2001b). Recently, condensed tannin accumulation was shown to occur in hybrid poplar (*Populus deltoids* x *P. nigra*) following exogenous jasmonic acid application (Arnold and Schultz, 2002), which supports the jasmonate-induced expression of *PtDFR* in this research. Thus, condensed tannin accumulation in aspen, as a response to

wounding and herbivore damage, may be triggered by an octadecanoid signal transduction mechanism that involves jasmonate signaling.

Overall, this research presents evidence that condensed tannin accumulation is a component of the systemic defense response in aspen. This suggests that phytochemical and protein-based defenses in aspen are induced by the same signaling system.

*4.6 Significance of this Research*

This research is the first to demonstrate that *DFR* is a herbivore-inducible defense gene in trembling aspen or any plant species. Trembling aspen is an excellent model for the study of condensed tannin biosynthesis, and *DFR* was successfully cloned from aspen and used as a tool to investigate the molecular aspects of condensed tannin synthesis. The cloning of *PtDFR* also allows for its use in new experiments, such as transgenic studies, which had not previously been possible. This study is the first to demonstrate that the induction of condensed tannins is correlated with the induction of *PtDFR* mRNA expression and DFR enzyme activity. Recently, condensed tannin accumulation has been shown to be induced by wounding and jasmonate treatment in poplars (Osier and Lindroth, 2001; Arnold and Schultz, 2002). Condensed tannin induction was confirmed, as concentrations of condensed tannins tended to be higher in wounded leaves compared to control leaves after 144 h, which is consistent with increases in *PtDFR* mRNA and DFR activity. Furthermore, induced condensed tannin synthesis appears to be controlled by the octadecanoid signaling pathway, as methyl jasmonate treatment induced the expression of *DFR*. The induced expression of *PtDFR* was comparable to the wound-induced expression of other known defense genes in aspen, suggesting that *PtDFR*

expression and condensed tannin accumulation is part of the local and systemic battery of defenses that aspen produces to deter herbivore feeding. This study shows an integration of protein-based and phytochemical-based defenses in aspen as defense proteins such as PPO and TI appear to be co-regulated with DFR and other phenylpropanoid enzymes, which are involved in induced condensed tannin accumulation. Overall, this research suggests that condensed tannins are an important inducible defense in aspen by being the first to show the underlying molecular biology of this induction.

*4.7 Suggestions for Future Research*

The cloning of *PtDFR* and its successful overexpression in *E. coli* allows for the elucidation of the substrate specificity of *PtDFR*. Extracts of *E. coli* expressing *PtDFR* can be used in DFR enzyme assays with different dihydroflavonols to determine if DFR has a substrate preference. Furthermore, *PtDFR-2* could be completely cloned and also expressed in *E. coli* to determine if its substrate specificity differs from that of PtDFR. To determine which amino acids are important in the substrate specificity of PtDFR, site directed mutagenesis could be conducted by changing various amino acids in the proposed substrate specificity region of PtDFR.

The importance of condensed tannins in defense against insects can be investigated by genetic manipulation of *PtDFR* expression in aspen. Both sense and antisense *PtDFR* plant transformation vectors were constructed and may be used in *Agrobacterium*-mediated transformation with the T89 (*Populus tremula* x *Populus tremuloides*) hybrid aspen clone, that was obtained and successfully micropropagated (see Appendix 2) (Nilsson *et al.*, 1992). A protocol for efficiently transforming the T89

aspen clone has been established using *Agrobacterium* (Nilsson *et al.*, 1992). Transformation with *PtDFR* expressed constitutively under the control of a duplicated 35S CaMV promoter in the sense orientation is expected to increase condensed tannins in aspen, while transgenic aspen expressing the antisense *PtDFR* controlled by the 35S CaMV promoter are expected to exhibit low concentrations of condensed tannins. These transgenic plants could be used in insect bioassays to determine if condensed tannin concentration can be correlated with insect performance. Normally, aspen contains quite high concentrations of condensed tannins, thus aspen plants with reduced tannin concentrations are expected to show increased susceptibility to insect damage.

Additionally, a transgenic approach could be taken to modify the structure of condensed tannins in aspen. In a previous study, condensed tannins in aspen were found to have a procyanidin:prodelphinidin ratio of 43:57 (Ayres *et al.*, 1997). This ratio could be modified with the cloning of the gene for flavonoid-3',5'-hydroxylase, which is responsible for hydroxylating the B-ring of the proanthocyanidin unit at the 3' and 5' positions, resulting in the synthesis of prodelphinidin units, which are the most highly hydroxylated and reactive units of condensed tannins. Transformation of a flavonoid-3',5'-hydroxylase gene expressed constitutively by the 35S CaMV promoter into T89 aspen is expected to result in transgenic aspen with a decreased procyanidin:prodelphinidin ratio in their condensed tannins, which is expected to result in the synthesis of condensed tannins that have increased hydroxylation. Condensed tannins that consist primarily of prodelphinidin units are expected to be highly toxic when consumed by insect herbivores because of their increased protein-precipitating capacity, and insects feeding on highly hydroxylated condensed tannins are expected to exhibit

slower growth rates and increased mortality compared to insects feeding on control aspen plants.

Finally, many transcription factors that are required for condensed tannin synthesis have recently been characterized from *Arabidopsis TT* mutants (Nesi *et al.*, 2000; Nesi *et al.*, 2001). Homologs of these transcription factors could be cloned from aspen and characterized to determine if wounding and herbivore damage increases the expression of the genes for these transcription factors. Induced expression of structural genes of the condensed tannin pathway are expected to be activated by the action of regulatory genes, and characterization of these regulatory genes could aid in the elucidation of the regulation of condensed tannin synthesis as well as the signaling mechanisms that control phenylpropanoid gene expression during the defense response.

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**Appendix 1: Recipe for Aspen Tissue Culture Medium (ACM)**

| | |
|---|---|
| Macronutrients (g $L^{-1}$ of medium) | |
| $NH_4NO_3$ | 0.40 |
| $Ca(NO_3)_2 \cdot 4H_2O$ | 0.556 |
| $K_2SO_4$ | 0.990 |
| $CaCl_2 \cdot 2H_2O$ | 0.096 |
| $MgSO_4 \cdot 7H_2O$ | 0.37 |
| $KH_2PO_4$ | 0.170 |
| Iron (g $L^{-1}$ of medium) | |
| Sodium-Ferric EDTA | 0.030 |
| Micronutrients (g $L^{-1}$ of medium) | |
| $MnSO_4$ | 0.0223 |
| $ZnSO_4 \cdot 7H_2O$ | 0.0086 |
| $H_3BO_4$ | 0.0062 |
| KI | 0.00083 |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.00025 |
| $CuSO_4 \cdot 5H_2O$ | 0.000025 |
| $CoCl_2 \cdot 6H_20$ | 0.000025 |
| Vitamins (g $L^{-1}$ of medium) | |
| Thiamine.HCl | 0.0001 |
| Nicotinic Acid | 0.0005 |
| Pyridoxine.HCl | 0.0005 |

Sugars (g $L^{-1}$ of medium)

| | |
|---|---|
| Sucrose | 20 |

For ACM-1 (bud break medium), add 0.1 g $L^{-1}$ lysine, 0.1 g $L^{-1}$ myo-inositol, 20 mg $L^{-1}$ adenine sulphate and 0.5 mg $L^{-1}$ 6-benzylaminopurine to above ACM medium (pH 5.5-5.6)

For ACM-2 (shoot induction, proliferation and growth medium), add 0.1 g $L^{-1}$ lysine, 0.1 g $L^{-1}$ myo-inositol, 20 mg $L^{-1}$ adenine sulphate, 0.5 mg $L^{-1}$ 6-benzylaminopurine and 0.02 mg $L^{-1}$ naphthalene acetic acid to above ACM medium (pH 5.5-5.6)

For ACM-3 (rooting medium), add 0.5 mg $L^{-1}$ indolebutyric acid and 0.04 mg $L^{-1}$ naphthalene acetic acid to above ACM medium (pH 5.5-5.6)

**Appendix 2: Recipe for Murashige and Skoog Medium (MS1)**

| | |
|---|---|
| Macronutrients (g $L^{-1}$ of medium) | |
| $NH_4NO_3$ | 1.65 |
| $KNO_3$ | 1.90 |
| $CaCl_2 \cdot 2H_2O$ | 0.44 |
| $MgSO_4 \cdot 7H_2O$ | 0.37 |
| $KH_2PO_4$ | 0.17 |
| Iron (g $L^{-1}$ of medium) | |
| $Na_2 \cdot EDTA$ | 0.03725 |
| $FeSO_4 \cdot 7H_2O$ | 0.02785 |
| Micronutrients (g $L^{-1}$ of medium) | |
| $MnSO_4$ | 0.0223 |
| $ZnSO_4 \cdot 7H_2O$ | 0.0086 |
| $H_3BO_4$ | 0.0062 |
| KI | 0.00083 |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.00025 |
| $CuSO_4 \cdot 5H_2O$ | 0.000025 |
| $CoCl_2 \cdot 6H_20$ | 0.000025 |
| Vitamins (g $L^{-1}$ of medium) | |
| Thiamine.HCl | 0.0001 |
| Nicotinic Acid | 0.0005 |
| Pyridoxine.HCl | 0.0005 |

| | |
|---|---|
| Glycine | 0.002 |
| Sugars (g $L^{-1}$ of medium) | |
| Myo-inositol | 0.1 |
| Sucrose | 30 |

An aspen hybrid (*Populus tremula* x *P. tremuloides*) clone (T89) was obtained from A. Seguin (Laurentian Forestry Centre, St. Foy, Quebec, Canada) for future transformation experiments. This clone was subcultured according to Nilsson *et al.* (1992) by sectioning an aseptic rooting culture into approximately 15-mm segments, avoiding the nodes where possible. Sections were placed on shoot initiation medium (MS1) containing 0.1 μg $ml^{-1}$ IBA, 0.2 μg $ml^{-1}$ BAP and 0.001 μg $ml^{-1}$ thidiazuron, and maintained in a University of Alberta environmental growth chamber (Environmental Growth Chambers, Chagrin Falls, Ohio). Shoots were transferred and maintained on shoot elongation medium (MS2), which is the same as MS1 medium, but without the thidiazuron. Rooting was induced by placing shoots on half-strength MS1 medium without hormones. In preparation for potting, plants were acclimated and planted as described in the Materials and Methods.

**Appendix 3: Sequence Data for Aspen Phenylpropanoid Genes**

Phenylalanine ammonia-lyase (*PAL*)

ATAATCCAACTTGGATTCCTGCTAGCAGTGAGATTCGATGGCAATCCATTGTT
GTAGAAATCATTTACGAGCTCACTGAACTGAGCAAATAGCAGCTTGCCTATT
GATGCAATAGCCAAACGTACATTATCCATTGAGACTCCAATTGGAGTCCCTG
GAAGTTACCACCATGTAAAGCCTTGTTCCTTGAAACATCAATTAGAGGGTTGT
CGTTCACTGAATTAATCTCTCTTTCAATTGACTTGGTCGAGAACGGATTACTT
CAATCTGGGGACCAAGCCATTGGGGTGAATCCTTAGAGCATAGCGATCCTGC
TTTGGTTTCTGCAAGGGGTCCGTTTCATGCAACTTCTTAGCTGCCTTCATGTAT
GCGCTGCCATCTAAGATGTGCTCCATTATAGCTGCAGCCTCAATTTGACCTGG
ATGGTGCTTCAATTTGTGTGTCAAATGGTCAGTGAATTCNGGTTTACCATTCA
TAACCTCAGCAAAAATTGCTGACAGANCTCCGATAAGACGCAAGAACTTGTC
TCGAAAAAAAACATAGAACCANGCAGAACACTGCATTCCATTACTAGAGCAGG
CTCTTAGCTGCANTCAANATCANATCATGCACTGCTTTAAGCTCAGCACTCNA
ACNTTCTCCGTGGACAGTGGCTGGAATNGGCTGCNGTCGTATCACAATGTNG
ATATGACTAATCCCTAAACATGATGTGCTCTATGCAGCTGATATNTNTTATGA
GACTGGTATACTCCAAATCAATCGAGC

4-Coumarate:CoA ligase (*4CL*)

TGTTTCACATCTTTTCGTTGAACAGCGTGTTGTTATGCTCGTTGAGAGCCGGTT
CTGCTGTTCTTTTAATGCAAAAGTCTGAGATAGCGATCACTGCTAGAGCTCAT
TCAGAAACACAATGTTTCGGTTGCGGCTGTGGTGCCACCACTGGTGCTGGCGT
TGGCCAAGAACCCATTGGTGGCGAATTCGACTTGAGTTCGATCAGGGTAGTC
CTGTCAGGGGCTGCCCACTGGGGAAGGACTCGAGGACCCTCAGGAGCAGGTC
CCAGCACCTGGACGGTTTGGATACAGCGGGCGTCACATTCTACTTCAACACC
TTCCACCATTGTCTGTGACGTGTAAACCCACCAGTCTACCTAACGTCCTCTCT
TGGTACACACACTGTATTCTCTGATCCAACTAAGACTTATACCGAAACTAGTT
GCCCCCTGAATTAGTCTTCACACACACAAT

Chalcone Synthase (*CHS*)

GGGCTGCACCGAATTCGAGCGATTAAAGATGGGGGCAGCCAAAATCCAAGA
TCACCCACCTTGTATTCTGTACAACCTCAGGTGTAGACATGCCAGGTGCTGAC
TACCAGCTCACCAAGCTCCTCGGCCTGCGTCCCTCCGTAAAGAGACTCATGAT
GTATCAGCAGGGCTGCTTCGCCGGCGGGACCGTCCTTCGTTAGCTAAGGACTT
GGCTGAGAAAACAAGGGCTCACGCGTTCTTGTTGTCTGCTCTGAAATCACTGC
TGTCACTTTTCGTGGACATCTGATATCCTTGGATCATGGTCGTCACGTTTGGC
AGGTCTCACATTTTTAGTCGATCGAATCGTTACTCATTCCGTGTTACTCCAACT
CTTCGATCGACGGCCARCGATGAACTTACTAGTGGTTACTTCACGCTAAGGAT
TCCTGGCTAATCCAAACT

Leucoanthocyanidin Reductase (*LAR*)

CATGGCATCCCAGTTGACCAAAAAGACAGCTTGTGTGATCGGCGGCTCAGGG
TTTGTGGCGTCTTTGCTTGTGAAATTGTTGCTTGAGAAGGGCTATGCAGTGAA
CACCACAGTCAGGGACCCAGACAATCAAAAGAAGGTTGCTCAAATAATAGC
GCTACAAAATCTGGGGGACCTAAACATATTCGGAGCAGATTTGACTGATGAA
GAGAGCTTCAATGCTCCCATAGCACGCTGTGAGCTTGTCTTCCAAGTTGCAAC
ACCAGTTAACTTTGCTTCTGAGGATCCTGAGAATGACATGATCAAGCCAGCA
ATTCAAGGAGTAC

Anthocyanidin Synthase (*ANS*)

GAGTGGGACGACTACTTTTTCCATCTCATTTTCCCCGAGGACAAGCGTGACAT
CTCCATATGGCCCAAGACACCTAGTGATTATACGAAAGTCACTAGTGAATAT
GCAAGGCTGCTGAGAATTTTAGCGAGCAAAATTCTGTCAGCACTATACACTT
GGCCTGGGATTGGAGGAAGGAAGGCTAGAAAAGGAGGTCGGTGGTATGGAA
GAGTTGCTACTTCAAATGAAGATCAACTACTACCCAGGTGCCTCAACCAGAC
TAGCTCTAGGTGAACTCACACGAT